

Adiponectin receptor expression and functional role in cultured cells

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Abstract

Hepatoma cells (HepG2 cells) were incubated for 24 h with different saturated fatty acids at different concentrations to examine a possible regulation of mRNA expression of adiponectin receptor 1 and 2. The results showed that incubation with saturated FAs had a tendency to reduce the expression of adiponectin receptor 1 in HepG2 cells, whereas no change was observed for adiponectin receptor 2. The fatty acid analogues TTA, TTTA and α TTA did not reduce the amount of either adiponectin receptor 1 or 2.

Adiponectin receptor 3, T-cadherin, was cloned, and the expression of T-cadherin in a selection of tissues was studied. The expression of T-cadherin was highest in fetal brain, heart, uterus, lung whole and skeletal muscle, whereas the amount in liver was barely detectable.

Experiments with human myotubes were performed to examine if incubation with full-length, globular and trimer adiponectin altered oxidation of fatty acid (oleic acid) or if trimer adiponectin altered glucose oxidation. Myotubes from three different donor groups, lean, obese and T2D, were used in these experiments. Trimer adiponectin showed a tendency to increase glucose oxidation. Globular adiponectin showed a tendency to increase oleic acid oxidation more than full-length adiponectin and trimer adiponectin.

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Abbreviations

AMPK	AMP-activated protein kinase
AR1	adiponectin receptor 1
AR2	adiponectin receptor 2
ASM	acid soluble metabolites
α TTA	alpha methyl tetradecyl thioacetic acid
BSA	bovine serum albumin
CAR	cell associated radiolabel
DMEM	Dulbecco's modified Eagle's medium
FFA	free fatty acids
flAdn	full length adiponectin
gAdn	globular adiponectin
HepA	heptadecanoic acid
LaA	lauric acid (12:0)
LB	Lauria-Bertani
LDH	lactate dehydrogenase
MA	myristic acid (14:0)
MEM	minimal essential medium
mRNA	messenger ribonucleic acid
OA	oleic acid (18:1, n-9)
PA	palmitic acid (16:0)

PBS	Phosphate Buffered Saline (Dulbecco's with Calcium Chloride and Magnesium Chloride)
RPL27	ribosomal protein 27
RT-PCR	Reverse transcriptase- polymerase chain reaction
StA	stearic acid (18:0)
tAdn	trimer adiponectin
T-cad	T-cadherin receptor/adiponectin receptor 3
TG	triglycerides
TTA	tetradecyl thioacetic acid ($\text{CH}_3\text{-(CH}_2\text{)}_{13}\text{-S-CH}_2\text{-COOH}$)
TTTA	triple tetradecyl thioacetic acid

1. Introduction

1.1 Adiponectin

Adiponectin is a hormone that is secreted by differentiated adipocytes and bone-forming cells (1). Plasma concentrations in humans range from 3 to 30 $\mu\text{g/mL}$ and are inversely proportional to body fat content. Adiponectin is the most abundantly secreted adipokine, making up to 0.01% of circulating protein in serum (2). The protein consists of 244 amino acids (30 kDa), is related to the complement 1q family and contains a carboxyl-terminal globular domain and a collagenous domain at the amino terminal (2). Circulating adiponectin levels are decreased in obese and insulin resistant subjects and the levels seems to decrease in parallel with progression of insulin resistance and type 2 diabetes. Elevated plasma adiponectin is strongly and independently associated with reduced risk of developing type 2 diabetes in healthy individuals (3). Adiponectin is encoded by the APM1 gene which maps to chromosome 3q27, a region identified as a susceptibility locus for metabolic syndrome and type 2-diabetes (3).

Adiponectin was originally identified by four independent groups using different experimental approaches and has therefore also been called Acrp30, GBP28, apM1 and AdipoQ in the past (4-6). Two different adiponectin receptors have been identified, the adiponectin receptor 1 (AR1) that is ubiquitously expressed in the body, and the adiponectin receptor 2 (AR2), whose expression is more restricted to skeletal muscle and liver. A third adiponectin receptor, T-cadherin, was later identified. The AR1 exhibits high affinity for globular adiponectin and low affinity for full-length adiponectin (7). The AR2 shows intermediate affinity for both types of adiponectin. The identification of AR1 and AR2 has helped us to gain a better understanding of the effects of adiponectin in different tissues.

Adiponectin displays a wide range of effects, including insulin sensitivity, activation of peroxisome proliferator activator receptor α (PPAR α) ligand pathways and AMP protein kinase (AMPK), and anti-atherosclerotic effects. Down-stream of the adiponectin receptors activation of AMPK followed by inhibition of acetyl coenzyme A carboxylase (ACC) and stimulation of PPAR α is essential for the effect of adiponectin (fig 1.2). Activation of AMPK results in stimulation of proteins involved in fatty acid (FA) transport and oxidation,

and an increased glucose uptake in muscle. PPAR α activation causes increased FA oxidation in muscle and liver and increases the fasting response in liver. The adiponectin gene contains response elements for peroxisome proliferator activator receptor γ (PPAR γ), a key regulator of glucose and lipid metabolism. Total energy expenditure, especially in hyperinsulinaemic conditions, increase with elevated serum adiponectin levels (2).

Adiponectin increases AMP-activated protein kinase activation and FA oxidation, and plays a central role in the regulation of food consumption. In the lean state adiponectin induces FA uptake into storage depots. When adipocytes reach a certain size, secretion is switched off to prevent further lipid accumulation (2).

Regulation of physiological responses to elevated serum adiponectin is complicated and occurs at several levels, including formation of oligomers, enzymatic cleavage and availability of its receptor isoforms. Adiponectin circulates in plasma as three distinct oligomers, trimers, hexamers (low molecular weight species) and high molecular weight (HMW) species. It may exist as full-length or smaller, globular fragments. However, almost all adiponectin exists as full-length adiponectin in plasma (8). An important function of HMW adiponectin in insulin sensitivity was suggested in 2001 by three independent groups (8). The hypothesis was that HMW adiponectin may be converted into a biologically active form in response to metabolic challenge, through reduction and proteolytic cleavage (3).

Adiponectin promotes oxidation of FA in muscle and synthesis of different adiponectin receptors in liver and other tissues, thus decreasing tissue triglyceride (TG) content and upregulation of insulin signaling. Increased tissue TG content can interfere with insulin-stimulated phosphatidylinositol (PI) 3 kinase activation and subsequent glucose transporter 4 (GLUT4) translocation and glucose uptake. This may lead to insulin resistance in muscle, and a decrease in TG by the action of adiponectin through its receptors that may contribute to improved insulin signal transduction (8).

T-cadherin (T-cad) is the third adiponectin receptor, and has also been called H (heart)-cadherin and cadherin-13 (CDH13). It is an atypical glycosylphosphatidylinositol-(GPI) anchored extracellular protein, member of the cadherin superfamily (9). T-cad is a receptor for hexameric and HMW forms of adiponectin, but not for the trimeric and globular adiponectin. Low-density lipoproteins (LDL) interfere with the normal function of T-cad because of binding to the receptor in endothelial cells (10). Only eukaryotically expressed

adiponectin bind to T-cad, implying that posttranslational modifications of adiponectin are critical for binding (3). T-cad is widely expressed in brain and the cardiovascular system, and lower levels are found in muscle. T-cad is expressed in low amounts in liver (10) and its tissue distribution is differing from that of AR1 and AR2. T-cad is regulated in a complex manner indicating a role in hormone and drug-induced changes in bone tissue. Adiponectin levels have been shown to be inversely associated with bone mineral density (10). T-cad has been shown to be involved in fracture healing (10). The function of T-cad is not well understood, but it may act as a co-receptor for an as yet unidentified signaling receptor through which adiponectin transmits metabolic signals (11).

T-cad regulates growth of cells in the nervous system. It was reduced in a glioblastoma cell line and reexpression of T-cad induced growth arrest (11). T-cad appears to be a negative regulator of cell growth, because it regulates motor axon projection in the embryonic nervous system via contact inhibition (10). T-cad is also found to protect endothelial cells from oxidative stress-induced apoptosis through activation of PI3K/Akt/mTOR survival signal pathway and suppression of the p38 MAPK proapoptotic pathway (9).

There is a potential for adiponectin replacement therapy in insulin resistance and related disorders if looking at the beneficial effect of adiponectin observed in animal studies. There is a co-variation in the amount of fat tissue and the amount of circulating adiponectin, where increasing amount of fat tissue reduces the amount of adiponectin and thus worsen the insulin resistance (3). Knock-out of the adiponectin gene in mice is associated with insulin resistance and low mitochondrial content and reduced mitochondrial enzyme activity in skeletal muscle (12). Acute adiponectin incubation in mice decreases insulin resistance, plasma FFAs and the triglyceride content of muscle and liver, and increases expression of genes involved in FA oxidation and energy expenditure (13). Glucocorticoids have been shown to decrease serum adiponectin levels in normal subjects treated with hydrocortisone and in patients with Cushing's syndrome (10). It has been proposed that increasing the expression of AR1 and AR2 may improve insulin sensitivity and reduce the risk of developing T2D (14).

Adiponectin's anti-atherogenicity might lead to the development of new therapeutic strategies for cardiovascular disease, diabetes mellitus and even the metabolic syndrome. Given the high plasma levels of adiponectin in humans, direct administration of adiponectin

protein to individuals with chronic disease as a type of therapy might be difficult. The reason for this can be that the underlying mechanisms that cause problems in maintaining high plasma levels of adiponectin are still present. The search for enhancers of endogenous adiponectin synthesis or a method for reducing the clearance of adiponectin might be the most practical way for therapeutic application related to adiponectin. Thiazolidinedione derivatives have been shown to potently increase adiponectin synthesis (15).

Myotubes

Myotubes grown from satellite cells have been used as an *in vitro* muscle cell model. Experiments have been performed with human myotubes grown from muscle biopsies from three different donors. The myotubes have been preincubated with adiponectin and later incubated for 4 h with [1-¹⁴C]oleic acid containing medium (fig 1.1).

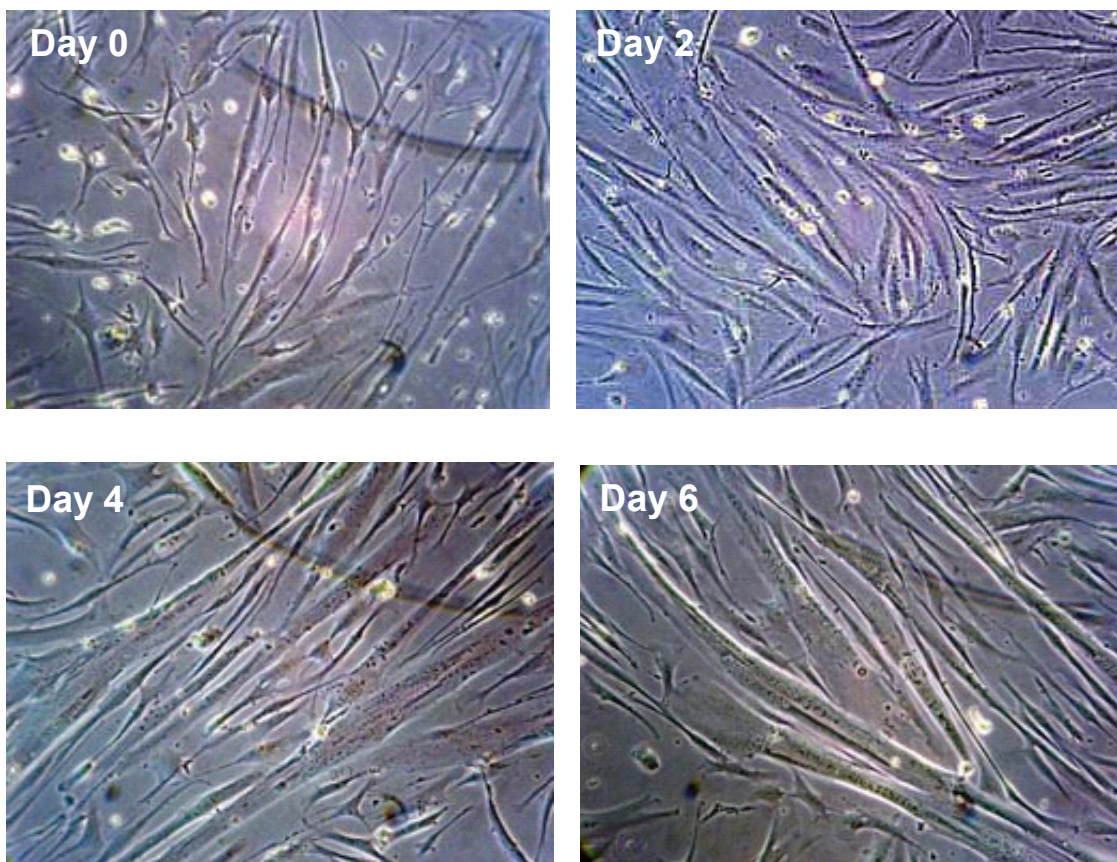


Figure 1.1 Photographs (x20) of differentiation of satellite cells to myotubes. Morphological appearance (phase contrast microscopy) during differentiation of a human satellite cell culture at day 0, 2, 4 and 6 after start of differentiation in insulin containing differentiation medium (pictures by Vigdis Aas).

Adiponectin and the mechanism of insulin sensitizing effect

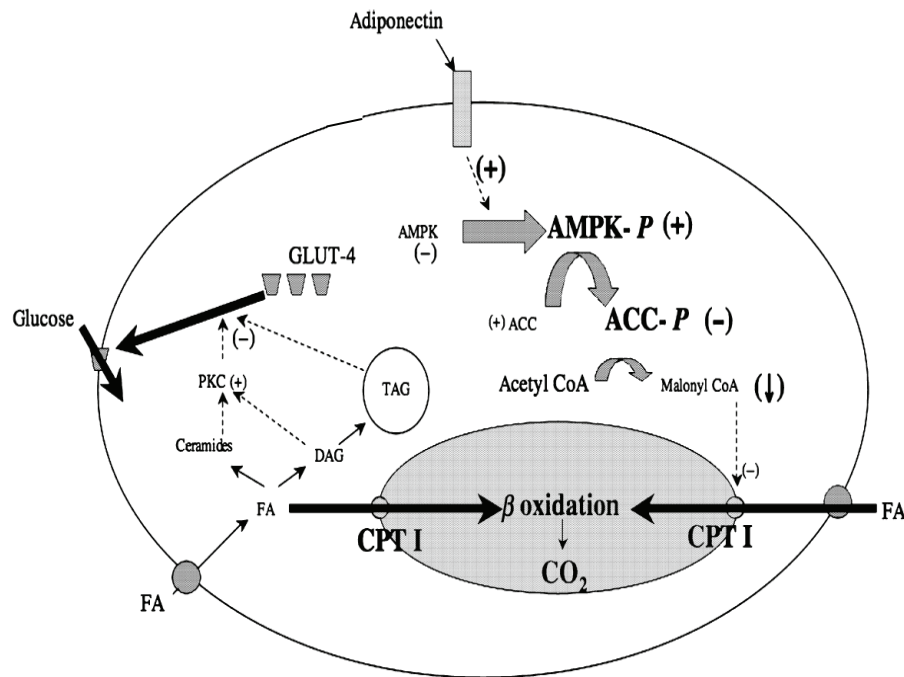


Figure 1.2 Adiponectin related to an insulin sensitizing effect. The proposed mechanism by which adiponectin stimulate FA oxidation and reduce lipid accumulation (16). There is a strong correlation between abnormal fatty acid (FA) metabolism and the development of insulin resistance in skeletal muscle. Globular adiponectin interacts with AR1 in skeletal muscle. Adiponectin stimulates phosphorylation of AMPK and ACC, and stimulates/increase FA oxidation and decrease TG storage in muscle. FA inhibits the translocation of GLUT-4 to the cell membrane and this result in a reduction in glucose uptake (16).

1.2 Adipose tissue and free fatty acids

Circulating free fatty acids (FFAs) derived from adipocytes are elevated in many insulin resistance states. Increased lipolysis in adipocytes due to poor effect of insulin, results in increased FA release from adipocytes. An elevation in circulating FA in obese patients results in increased uptake of FA and excess deposition of lipids within the muscle cell if the FA cannot be oxidized (16). The FAs are stored in muscle cells instead of adipose tissue and interfere with the normal function. Visceral fat is less sensitive to insulin than subcutaneous fat. This causes direct flux of adipocyte derived FAs through the portal vein into the liver, and can stimulate glucose production, thus providing a signal for both insulin action and insulin resistance in the liver (13). Although insulin does not stimulate hepatic glucose uptake, it blocks/inhibits glycogenolysis and gluconeogenesis, and stimulates glycogen

synthesis, thus regulating fasting glucose levels (13). Elevated FFAs might cause accumulation of fat in muscle, liver and/or β -cells, which may interfere with different metabolic pathways and signalling (17). Insulin resistance is associated with impaired skeletal muscle oxidation capacity and reduced mitochondrial number and function. Lipid oversupply results in change in Randle glucose fatty acid cycle (fig 1.3), alter membrane lipid composition, promote triglyceride accumulation, increase ceramide biosynthesis, increase hexosamine biosynthesis and interact with insulin signalling and glucose disposal (18). Skeletal muscle has been considered one of the primary targets for understanding mechanisms for development of T2D and insulin resistance, mainly because it is the most important glucose absorptive organ.

Randle glucose-fatty acid cycle and insulin resistance

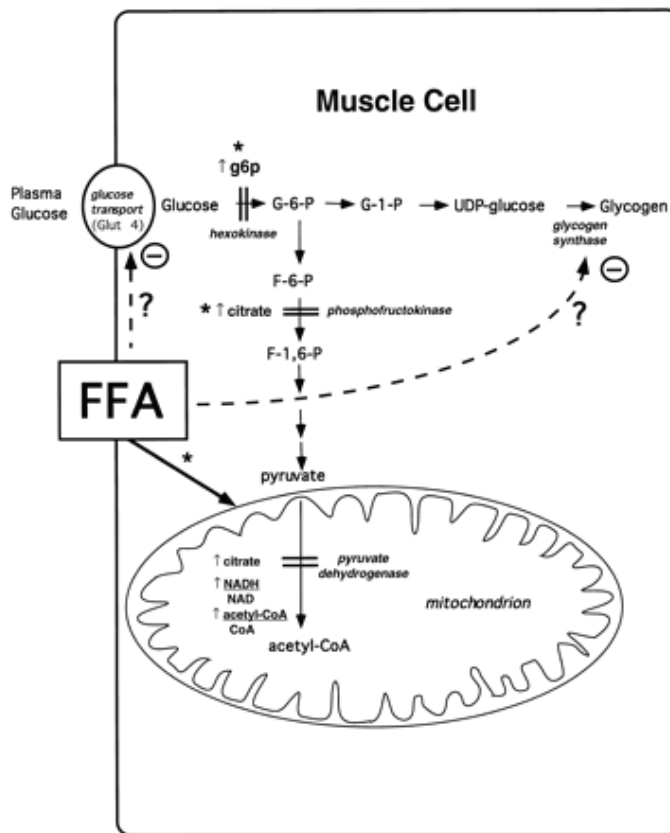


Figure 1.3 Schema of potential sites of free fatty acid action on insulin mediated glucose metabolism in skeletal muscle and those sites (*) hypothesized to be affected by Randle et al (18). G-6-P; glucose-6-phosphate; G-1-P, glucose-1-phosphate; F-6-P, fructose-6-phosphate, F-1,6-P; fructose 1,6 biphosphate. Free fatty acids induce insulin resistance in humans by initial inhibition of glucose transport/phosphorylation. This is then followed by an approximately 50% reduction in both the rate of muscle glycogen synthesis and glucose oxidation. A reduction in carbohydrate oxidation is responsible for one-third, while impairment of non-oxidative glucose metabolism (mostly glycogen synthesis) accounted for two-thirds of the fatty acid-dependent decrease in glucose uptake (18).

1.3 Aims

The purpose of this project was to study the effects of incubating hepatoma (HepG2) cells with different fatty acids (FAs), and measure expression of adiponectin receptor (1 and 2) mRNA.

To clone the third adiponectin receptor, T-cadherin, and study its expression in a tissue panel and in several cell lines.

To study the effects of incubating HepG2 cells with different FAs and measure expression of T-cadherin receptor mRNA.

To study chronic and acute effects of full-length, globular and trimer adiponectin on oleic acid oxidation and trimer adiponectin on glucose oxidation, in human myotubes.

2. Materials and Methods

2.1 Materials

Dulbecco's modified eagle medium (DMEM), fetal calf serum (FCS), penicillin-streptomycin 10000 U/mL-10000 mg/mL was obtained from Gibco, Paisley, UK. Ultroser G was obtained from BioSeptra, Process Division of CIPHERGEN Biosystems Inc, Paris, France. [1-¹⁴C]sodium bicarbonate, [6-¹⁴C]glucose and [1-¹⁴C]oleic acid were obtained from American Radiolabel Chemical Inc, MO, USA. Insulin Actrapid was obtained from Novo Nordisk, Bagsværd, Denmark. Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad Laboratories, Oslo, Norway. LDH assay Cytotoxicity Detection Kit and TaqMan Reverse Transcription Reagents were obtained from Roche Diagnostics, Mannheim, Germany. 12-well cell culture cluster plates were obtained from Corning Incorporated-Costar, NY, USA. 96-well plates were obtained from Greiner Bio-One, Frickenhausen, Germany. PCR, PfuUltraTMHigh-Fidelity DNA Polymerase was obtained from Stratagene, TX, USA. Reverse Transcriptase; Gene Amp®PCR System 9700 was obtained from Applied Biosystems international, USA. Agarose gel was obtained from SeaKem LE Agarose-Cambrex Bio Science, Rockland, USA. QIAquick gel Extraction kit was obtained from Qiagen, Hilden, Germany. pEntr Directional TOPO cloning kit and TRIzol Reagent were obtained from Invitrogen, Carlsbad, USA. Jetquick Plasmid Miniprep and Maxiprep were obtained from Genomed, Löhne, Germany. All reagents for cell culture, L-carnitine and bovine serum albumin-essentially FA free were obtained from Sigma Chem. Co, St.Louis, MO, USA. All other chemicals used were standard commercial high purity materials.

2.2 HepG2 cells

Human hepatoma cells, HepG2, were obtained from the American Tissue Culture Collection (ATCC, No HB-8065, Manassas USA). The experiments that were performed with these cells were a continuation of previous experiments (19;20).

The cells were cultured in minimal essential medium (MEM, M2279) containing 1 g/L glucose, supplemented with 10 % heat-inactivated fetal calf serum (FCS, F7524), 10.000 U/mL penicillin, 10 mg/mL streptomycin (P/S, P4333), 200 mM L-glutamine (G7513), 100X non-essential amino acids (M7145) and 11 mg/mL sodium pyruvate (S8636).

The cells were grown in biocoat flasks in an incubator maintaining controlled, constant temperature (37°C) and CO₂ enriched atmosphere (5 %). The wells/flasks contained approximately 1 ml medium per 10 cm². The medium was changed every 2-3 days until the cells reached confluence.

Trypsination was performed using 0.25 % sterile filtered trypsin EDTA solution. Old medium was removed and the wells were washed twice with 1 ml trypsin before they were added 3 ml trypsin and put in the incubator holding 37°C for 5 -15 min until they detached.

The cell suspension was transferred to a 50 ml falcon tube and 6 ml of the cell medium were added to inactivate the trypsin's action. The suspension was centrifuged at 13000rpm for 3 min at room temperature. The supernatant was discharged and the cell pellet re suspended in DMEM medium and the cell concentration determined by using a counting chamber, hemocytometer. The suspension was then diluted and the cells sub cultured into new biocoat flasks, or 12 well plates for experiments.

2.2.1 Experiments with HepG cells

The cells were seeded into 12 well cells plates approximately 7 days before experiments were performed. For adiponectin receptor regulation studies, the cells were given different fatty acids (FAs) for various time periods, usually 24 h.

The FAs were diluted with DMEM medium to concentrations of 200, 100 and 50 µM, before they were added to the HepG2 cells. Two parallels of each concentration of the different FAs were added to the wells and the cells were incubated 24 h before they were harvested. The cell medium was removed and a LDH (lactate dehydrogenase) assay was performed to assess plasma membrane damage level. The method was performed according to instruction manual. 50 µl of the cell medium was transferred to a 96-well plate with two parallels of each tube. Reaction mixture was prepared shortly before use, as described in manual, and was added to the cells. The plates were covered by aluminum foil. Bobbles in the wells were

removed, and the plates were stored in an incubator at 37°C for 30 min. For a measure of the level of 100 % cell lysis, diluted Triton was added. 50 µl LDH reagent was added to each well.

The result was detected by a spectrophotometric microplate reader (Titertek Multiscan Plus (MK-II), LabSystems, Helsinki) at 492nm.

2.2.2 RNA isolation with TRIzol

The RNA from the cells was isolated using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA), maintaining the integrity of RNA, while disrupting cells and dissolving cell components. All tubes containing RNA were kept cool on ice to avoid room temperature RNase degradation. Each well was added 0.5 ml TRIzol Reagent, scraped with a cell scrape and lysate from two wells was transferred to 1.5 ml Eppendorf tubes. At this stage the lysate was stored at -70°C if not processed right away.

The tubes were kept at 30°C for 5 min and then added 0.2 ml chloroform. This was shaken hard in horizontal position for at least 15 seconds and then kept 2-3 min at room temperature. Then the tubes were spun 15 min 13000 rpm 4°C and put in a rack. The separated clear upper phase consisting of water and RNA was transferred to 1.5 ml Eppendorf tubes on ice. If the upper phase was contaminated by mistake, the tubes were re-centrifuged. RNA was precipitated by adding 0.5 ml isopropanol to the 1.5 ml Eppendorf tubes. The tubes were vortexed, let standing 10 min RT, spun 10 min 13000 rpm 4°C and then the supernatant was discarded. Pellet was washed by adding 1 ml 75 % ethanol in DEPC-water then vortex, spun 5 min 8000 rpm 4°C and all supernatant discarded, leaving pellet almost dry. Then the pellet was air dried 5-10 min (before pellet was getting invisible) then resolved in 20 µl DEPC-water, pipetted up and down, let standing for 10 min in 60°C, vortexed, spun down and 1 µl was removed for Optical density (OD, 1/100X) measurement. The RNA was stored at -70°C.

2.2.3 Measure RNA concentration

RNA quality and quantity were determined spectrophotometrically based on optical density (OD) measurements on the Heliosγ (Thermo Spectronic, Cambridge, UK). 1 µl of each sample was diluted in 99 µl DEPC-water. OD 260nm (OD 260nm=1=40 ng/µl) was

measured and concentration calculated. The RNA was diluted to a concentration 0.3 µg/µl and the samples were prepared for reverse transcriptase.

Alternatively, the Nanodrop method was used, eliminating the need of pre-diluting sample.

2.2.4 Reverse Transcription, RT-PCR

TaqMan Reverse Transcription Reagents (Roche diagnostics, Mannheim, Germany) were used to convert a maximum of 2 µg total RNA to cDNA. Random hexamers were used (appendix).

All non-enzymatic components were combined and vortexed briefly. Enzymatic components were added. 28 µl of the mixture was transferred to glass capillaries, and 2 µl of the different samples of RNA (0.3 µg/µl) was then added. The tubes were capped and centrifuged to collect liquid at the bottom.

The instrument used was Touchgene Gradient machine (Cambridge, UK).

Thermal cycling parameters were incubation 25°C for 10 min, reverse transcription 48°C for 30 min and reverse transcriptase inactivation 95°C for 5 min. A final hold at 4°C for samples was obtained. Obtained cDNA was stored at -20°C.

2.2.5 Real Time Polymerase Chain Reaction, RT-PCR

Target gene LightCycler Kit (FastStart DNA Master SYBR Green I) and master mix supplied in kits from Roche Diagnostics (Mannheim, Germany) was used for amplification and detection of RNA. Primers were provided from Eurogentech (Seraing, Belgium).

Adiponectin receptors 1 and 2:

(5'-CTTCTACTGCTCCCCACAGC-3' and 5'-GATAGCGACTCCCGAAACAG-3') and (5'-CAACTGGATGGTACACGA-3' and 5'-AGAACGTACCTGTGGC-3').

Housekeeping gene RPL27:

(5'-CCTACAGCCATGCTCT-3' and 5'-CATCCTTATTGACGACATG-3').

Duplicate PCR reactions were performed in narrow glass capillaries with a reaction volume of 20 μ l that include 2 μ l cDNA. DEPC water was added instead of cDNA to one of the capillaries as a negative control. RPL27 was used to prove the integrity of the RNA and was tested in parallel to AR 1 and 2.

The PCR program started with a denaturizing step at 95°C for 10 min, then temperature cycling was initiated and consisted of denaturizing at 95°C for 10 sec, hybridization at 55°C for AR1 and RPL27 and 60°C for AR2 for 3 sec and elongation at 72°C for 10 sec. A final hold for 30 sec at 40°C was obtained. Approximately 45 cycles were performed.

2.3 Cloning of human adiponectin receptor 3

For the cloning of human adiponectin receptor 3 (T-cadherin, CDH13), a blunt-end PCR product was cloned directionally into the pENTRTM D-TOPO entry vector.

The primers hTCad-CDS-L (5'-CAC-CAT-GCA-GCC-GAG-AAC-TCC-GCT-CGT-3') and hTCad-CDS-R (5'-TCA-CAG-ACA-AGC-TAA-GCT-GAA-GA-3') from Eurogentec (Seraing, Belgium) were designed based on the mRNA sequence of CDH13 (GeneBank, NCBI sequence viewer) to amplify the translated region (121bp-2262bp) with use of PfuUltraTM High-Fidelity DNA Polymerase-kit (Stratagene, TX-USA), according to manufacturer's instructions with some modifications:

The total reaction volume for the amplification reaction was 50 μ l: 36.6 μ l MQ-water (Millipore, Bedford, USA), 5 μ l 10X buffer that comes with enzymes, 4 μ l dNTPs (4x2.5mM/10mM total), 1 μ l cDNA as template (nuclease free water was used as a negative control), 1 μ l primer hTCad-CDS-R (25 μ M) 1 μ l primer hTCad-CDS-L (25 μ M) and 1 μ l PfuUltra High-Fidelity DNA polymerase (2.5 U/ μ l). Total RNA from heart (Human total RNA master panel II, BD bioscience) was first Reverse Transcribed to cDNA and then used as template. The use of heart cDNA gave a single band monitored by agarose gel electrophoresis 1% (110 V 90 min. Size of the band 2141 bp). Monocyte total RNA U937 (ATCC CRL-1593.2TM, Manassas USA) was first Reverse Transcribed to cDNA and then used as template, but had to be replaced with heart cDNA (data not shown).

The instrument used was Gene Amp PCR System 9700 (Applied Biosystems, USA).

Thermal cycling parameters were initially denaturizing 95°C for 2 min, 95°C for 30 sec, 55°C for 30 sec, 72°C for 2 min and 30 sec, for approximately 30 cycles and a final elongation 72°C for 10 min was obtained.

After the PCR reaction was completed 10 µl of the product was analyzed by agarose gel electrophoresis 1 %, with 1 kb Plus ladder (Invitrogen Life Technologies, Carlsbad, CA) as a measure of fragment size. The gel was run at 100 V for approximately 90 min. The desired product was 2141 base pairs (bp) and the size was confirmed by comparing the band with ladder bands of specific sizes.

The gel product DNA was purified by using QIAquick Gel Extraction Kit Protocol. This procedure was performed according to the manufacturer's instructions.

2.3.1 QIAquick Gel extraction

The DNA fragment was cut out of the agarose gel using a UV light plate. This piece was put into an Eppendorf tube and the weight was measured. QG buffer was added and the tube was incubated at 50°C in a water bath for 10 min, or until the gel piece was solved. The yellow solution was then transferred to a column in which maximal volume was 0.8 ml, with an Eppendorf tube underneath. The column was then centrifuged at 13000 rpm for 1 min.

The volume collected in the tube was discharged and 0.5 ml QG buffer was added to the column and centrifuged one more time at 13000 rpm for 1 min.

0.75 ml PE buffer was added to wash the column, let stand 2-5 min and then centrifuged for 1 min. The liquid in the tube was discharged and the column centrifuged one more time for 1 min.

The column was transferred to a new tube and 50 µl EB buffer (10mM Tris-Cl, pH 8.5) was added in the center of the column and centrifuged 1 min.

The elute was stored at -20 °C.

2.3.2 Measurement of DNA concentration

The concentration of DNA was measured with use of agarose gel electrophoresis 1% 100 V for 3 h and 1 kb Plus ladder as a measure of the size of the bands. The 2141-bp band contained approximately 8% of the mass applied to the gel. Dilution lines of the 1 kb Plus ladder was used and the concentration of DNA determined visually by comparing the size of the bands with the size of the different dilutions. The concentration of DNA was then calculated.

2.3.3 TOPO cloning reaction

The blunt-end PCR for hTCad was directionally cloned into the pENTRTM-TOPO vector and transformed into One Shot® chemically competent *E. coli*.

Cloning was obtained using a 0.5:1 or 2:1 molar ratio of PCR product and TOPO vector. This equals 5-10 ng of a 2 kb PCR product. The TOPO Cloning reaction was performed as described in the manual.

4.0 µl of the PCR product (1.33ng/µl), 1.0 µl of the salt solution and 1.0 µl of the TOPO® vector that is a topoisomerase I, were mixed gently and incubated for 5-30 min at room temperature to obtain sufficient amounts of colonies, which were put on ice afterwards or stored at -20°C overnight.

From this solution 2 µl was put into a vial of *E. coli*, mixed gently and incubated on ice for 5 min. Then the cells were heat shock treated for 30 sec at 42°C without shaking and immediately transferred to ice, added 250 µl of room tempered S.O.C. medium and incubated at 37°C for 1 hour in horizontally position while shaken at 200 rpm.

The cells were plated out on pre-warmed 10 cm LB (Luria-Bertani, blue/white selection) agar dishes containing 50µg/mL kanamycin. A volume of 50 µl and 200 µl from each transformation were spread on the agar dishes to ensure separately grown bacteria colonies, and the dishes were incubated overnight at 37°C.

The positive clones were analyzed by picking 5-10 single transformed (white) colonies and culturing them overnight in 5 ml LB medium containing 10 µl kanamycin (30 µg/µl).

The tubes were incubated at 37°C overnight in horizontally position while shaken (200rpm). To extract and purify the high copy plasmid DNA from *E.coli* cultures the Plasmid MiniPrep (JETquick) kit was used. The procedure was performed according to manufacturer's instructions.

2.3.4 Miniprep (JETquick)

A volume of 2 ml was removed from the bacteria suspension and centrifuged 13000rpm for 5 min. The supernatant was discharged and the pellet was resolved in 250 µl G1 solution (provided in the kit) to a homogenous solution. From solution G2 250 µl was added for lysis of the cells. The liquids were mixed carefully without vortexing, and let stand 5 min at room temperature. Then 350 µl of solution G3 was added to neutralize the lyses solution and mixed carefully and centrifuged at 13000rpm for 10 min. The Jetquick spin column was placed in a 2 ml microfuge tube and the supernatant transferred from the centrifugation process to the column and centrifuged at 13000 rpm for 1 min. The flowthrough was discarded and 500 µl G4 solution was added to wash the column. Then the column was centrifuged at 13000 rpm for 1 min. The flowthrough was discarded and the column centrifuged again. The plasmid was eluted by placing the column into a clean 1.5 ml microfuge tube and 75 µl DEPC water was added to the middle of the column and then centrifuged 13000 rpm for 2 min. The elute was stored -20°C. OD₂₆₀ measurements were performed from the elute to calculate the concentration of high copy plasmid.

2.3.5 Restriction enzyme digest

To ensure that the cloning products were the correct ones, a restriction enzyme digest analysis was performed. For the restriction enzyme digest, the restriction enzymes Not I (TAKARA BIO INC. Shiga, Japan) and Sal I (Promega, WI-USA) were chosen. Not I is a 0 cutter and Sal I is a 1 cutter (cuts with 1636/1640pb, and the longest bp sequence is 501bp). This procedure was performed according to Manufacturers instruction, where 9 tubes containing 0.5 µl Sal I, 1 µl Not I, 3 µl plasmid DNA, 2 µl buffer D and 13.5 µl MQ water were mixed to a total volume of 20 µl. The 10th tube contained 3 µl of plasmid DNA, 2 µl buffer D, 15 µl MQ water but no enzymes. After incubation at 37°C overnight, the samples were analyzed by agarose gel electrophoresis 1% (110 V, 90 min) to confirm the size of the PCR products. More DNA from positive clones was used in a new restriction enzyme digest

with bigger volumes. This was also analyzed by agarose gel electrophoresis. For the new restriction enzyme digest 10 µl buffer, 2 µl Not I, 2 µl Sal I and 10 µl DNA was mixed and the total reaction volume was 100 µl. This was put in an incubator keeping 37°C for 1-2 h, and afterwards analyzed by agarose gel electrophoresis 1% (100 V, 90 min). A match was found with one of the bands and the content of this bacterial suspension was chosen for further experiments.

100 ml LD-medium was added 200 µl kanamycin (30 µg/mL) and 100 µl from the bacterial suspension. This was put in a shaker holding 37 °C, overnight.

Plasmid DNA was extracted and purified from the gel by JETstar MaxiPrep procedure. The plasmid vectors obtained by MaxiPrep were control sequenced by MedProbe AS (Norway) in both forward and reverse direction by using M13 forward (GTAAACGACGGCCAGTC) and reverse (CAGGAAACAGCTATGAC) primers respectively. All base pairs in the insert were analyzed to assess if any mutations had occurred in the DNA during cloning process. This sequencing showed uncertain regions in one area of the DNA. These regions had to be sequenced one more time to assure that no mutations had occurred. This uncertain region was localized in the middle of the DNA.

A primer that covered this area was found after analyzing the base pair region with different primers that would cut and cover the desired area of the DNA.

The plasmid vectors were control sequenced one more time by MedProbe (Norway) with hTCAD-F primer (Eurogentec, Seraing, Belgium) (5'-GTT-GGC-AAG-GTA-GTC-G-3') to survey uncertain regions in middle of the DNA sequence.

2.3.6 Maxiprep (JETstar)

A column was put in the rack with a collective tube underneath and 30 ml of solution E4 was added to equilibrate the column. The solution was let to pass through the column without adding any pressure.

The bacterial suspension was transferred to a plastic tube and centrifuged 5000rpm at 4°C for 10 min. All traces of medium were carefully removed and 10 ml of solution E1 was added, the pellet was resolved to a homogeneous suspension and transferred to a glass tube.

To lysis the bacteria cells 10 ml of solution E2 was added and mixed carefully, not vortexed, and let stand for 5 min at room temperature.

To neutralize the lysis process 10 ml of solution E3 was added, mixed immediately to a homogenous solution was obtained, but not vortexed. The solution was centrifuged at 13000 rpm for 10 min at room temperature.

To load the column the supernatant from the last centrifugation was applied and the lysate allowed to migrate through by gravity flow. The column was washed with 60 ml of solution E5 and the plasmid eluted with addition of 15 ml of solution E6. A clean tube was placed underneath the column. The remaining solution was not forced through the column. Plasmid precipitation was performed by adding 10.5 ml isopropanol and centrifuged at 13000 rpm at 4°C for 30 min. The supernatant was removed and the plasmid DNA washed with 70 % ethanol. The column was re-centrifuged 13000rpm for 30 min. The pellet was air dried for 10 min, re-dissolved in 100 µl MQ water and transferred to an Eppendorf tube. The tube was washed one more time with 100 µl MQ water and this volume was added to the tube.

The concentration was measured by OD₄₉₂ nm and thereafter calculated.

A new bacterial culture in liquid media was made for storage. 1.5 ml of the bacterial suspension was added 0.5 ml glycerol 60% and stored at -70 °C.

2.3.7 Standard curve

For the purpose of quantification, a standard curve was systematically constructed from a serial 10-fold dilution of the cDNA generated during the TOPO-cloning reaction in DEPC-treated water. The concentrations used were in the range between 100 pg to 0.001 fg. The concentration used for further PCR was 0.01 pg. This concentration corresponded to the linear region and thus the number of cycles found in earlier T-cadherin experiments.

PCR was performed as for qRT-PCR of AR1 and AR2, using a LightCycler (Roche Diagnostics, Mannheim, Germany). The assay was performed in duplicate.

The primers used were hTCAD-F (5'-GTT-GGC-AAG-GTA-GTC-G-3') and hTCAD-R (5'-CGATGTAGGGGCCTTC-3'), both from Eurogentec (Seraing, Belgium).

A straight line was fitted to the data created from different concentrations of the serial dilution of T-cadherin cDNA. Linear regression of T-cad concentrations created a slope of -2.686 ($r = -0.98$) and indicated that the standard curve was valid and could be used for T-cadherin quantification.

The program used for PCR was initiated with a denaturizing step at 95°C for 10 min. The temperature cycling was then initiated and consisted of denaturizing at 95°C for 10 sec, hybridization at 55°C for RPL27 and 60°C for hTCad for 3 sec and elongation at 72°C for 10 sec. A final hold for 30 sec at 40°C was obtained. Approximately 45 cycles were performed.

2.3.8 1 Kb Plus DNA Ladder

The 1 Kb Plus DNA Ladder originated from Invitrogen Life Technologies (Carlsbad, CA) and the concentration was 1 µg/µl. It was used for verification of PCR product on agarose gel stained with ethidium bromide, electrophoresis 80-100V for approximately 90 min. The 1650-bp band contained approximately 8 % of the mass applied to the gel and when the size of the bands were compared to dilution lines of the ladder, the concentration could be calculated.

2.4 Scanning tissue panels for occurrence of T-cadherin receptor

A selection of tissues was scanned to gain knowledge about distribution of T-cadherin receptors in the body. The same human tissue panel was previously analyzed for the expression of adiponectin receptor 1 and 2.

This tissue panel was composed of tissue samples from adipose tissue, adrenal gland, bone marrow, brain whole, fetal brain, fetal liver, heart, kidney, liver, lunge whole, placenta, prostate, salivary gland, skeletal muscle, testis, thymus, thyroid gland, trachea, uterus and spinal cord. Measured values were normalized by use of RPL27 as an internal control. Duplicate test of these tissues were analyzed.

RNA from a selection of tissues like BeWo (placenta trophoblasts), THLE (liver), Raji and Ramos (B-cells), THP-1 (control-monocytt and PMA treated-macrophage), U937 (control-monocytt and PMA treated-macrophage), Jurkat (T-cells) and SGBS cells (undifferentiated

and differentiated) were isolated with TRIzol, the concentration was measured with OD and RT Taqman Reverse transcriptase was performed as the procedure described for HepG2 cells.

cDNA from a previous study with myotubes from 8 type 2 diabetic and 8 obese donors was analyzed for T-cadherin as well as AR1 and AR2 expression in human myotubes. Differentiated myotubes (4d) had been pre-incubated with bovine serum albumin (BSA) and different FAs in 100 μ M concentration; oleic acid (OA), eicosapentanoic acid (EPA) and tetradecylthioacetic acid (TTA) for 4 days, before harvested for RNA isolation and cDNA synthesis. cDNA from type 2 diabetic and obese donors was pooled into their respective groups and the average effect of different pre-incubations was analyzed to investigate the effect of the different FAs on T-cad receptor and AR1 and AR2 expression. Duplicate test of these tissues were analyzed.

Different cDNA was analyzed by PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany). RPL27 was used as the housekeeping gene.

The program used for PCR analyzes was initiated by a denaturizing step at 95°C for 10 min, the temperature cycling was then initiated and consisted of denaturizing at 95°C for 10 sec, hybridization at 55°C for RPL27 and 60°C for hTCad for 3 sec and elongation at 72°C for 10 sec. A final hold for 30 sec at 40°C was obtained. Approximately 45 cycles were performed.

2.5 Skeletal muscle cells

Primary muscle cells from biopsies:

Donor LD 16; male, 33 years old, fasting blood glucose levels 5.5 mM, BMI 22.99.

Donor LD 29; male, 35 years old, fasting blood glucose levels 4.9 mM, BMI 19.62.

Donor LD 30; male, 36 years old, fasting blood glucose levels 5.0 mM, BMI 26.20.

2.5.1 Seeding out muscle cells

Cryogenically frozen cells were quickly thawed. The cell suspension was then transferred to a 50 ml falcon tube containing M1-medium. The cyro-tube was washed with the same M1-

medium twice to assure that all the cells were transferred. The suspension was then mixed thoroughly before the cells were seeded out on 96-well plates by an 8-channel multi pipette (100 μ l per well inside 80 wells). CellBIND® plates were chosen to improve cell attachment. Cell density was targeted to approximately 3000-6000 cells/cm².

2.5.2 Growing and differentiation of primary cells into myotubes

The primary cells were grown in an incubator maintaining constant temperature at 37°C and 5 % CO₂ enriched atmosphere. The flasks contained approximately 1 ml medium per 10 cm². After 24 h of incubation the initial medium was changed to M2-medium. The cells were then proliferated until reaching 70-80 % confluence. The medium was then switched to M3, thus initiating differentiation of the cells into myotubes. The myotube poly-nucleated structures formed from fusion of precursor cells were to be apparent 1-2 day(s) after initiating differentiation. The M3-medium was changed every second day. The myotubes were usually given preincubations (if any) dissolved in M3-medium from day 4 after initiation of differentiation. The cells were visually inspected during growth and differentiation to see that they developed as intended. The myotubes were harvested between days 4 to 10 after initiating of differentiation.

Experiments with full length adiponectin

The significance of adding serum to the pre-incubation medium was tested in the preliminary experiment. The medium added to half of the wells contained 2 % fetal calf serum (FCS) and the remaining wells did not receive any FCS in their media. The effect of adiponectin was also assessed by adding 0.5 μ g/mL full-length adiponectin into the medium of half of the two serum incubations. The medium added to the acute incubation was serum deficient. Only one experiment was performed with full-length adiponectin.

2.5.3 Fatty acid oxidation

Medium was changed from M3-medium to experimental medium at different time intervals. Four wells were given same incubation sufficient for each different experimental condition, to get a good estimate of the mean ¹⁴CO₂ generated and trapped during a time interval. Four

wells received just regular M3-medium as a control. The effect of incubating cells with adiponectin on FA oxidation was investigated. The amount of FA oxidation was measured by using a CO₂ trapping device (fig 2.1).

For each time interval the cells received medium containing different amount of adiponectin. The first experiment was performed with full-length adiponectin (flAdn), later experiments were performed with globular (gAdn) and trimer type (tAdn). The cells were incubated for 4 h with radioactive oleic acid medium, followed by protein content, acid soluble metabolites, cell associated metabolites and CO₂ amount measurements. Wells receiving acute incubation were incubated with adiponectin in the medium during the 4 h long trapping period. The remaining cells were investigated for accumulated adiponectin effect on FA oxidation, and no adiponectin was added to the medium during trapping period.

The medium was devoid of H₂CO₃ buffer. The cells were washed with DPBS (with Calcium- and Magnesium Chloride) containing 20mM Hepes buffer before they were put inside the airtight CO₂-trapping device. A silicon mat was put between the two plates before they were sealed in the trapper device. The filter plate wells were pre-wetted with 25 µl 1 M NaOH shortly before they were sealed.

Acutely, the cells were given medium containing [1-¹⁴C]oleic acid, concentration 1 µCi/mL (50µM) and total FA concentration 18.2 nmol/mL. The device was put into an incubator maintaining 37°C for 4 h.

After incubation, the cell medium was transferred to another plate and the cell plate was washed twice with 200 µl PBS, and then 0.05 M NaOH was added and the plates were covered with plastic and cooled at -20°C, if not processed right away.

The cells were assayed for protein, cell-associated radiolabel (CAR) and acid soluble metabolites (ASM). The wells were added Microscint-O or Optiphase Supermix, the plate was sealed on top with translucent plastic adhesive cover slip and let stand for 24 h. All samples were analyzed in the Microbeta triluX® from Wallac, either in opaque 96 wells filter plates or in opaque 96 wells plates with transparent base plate.

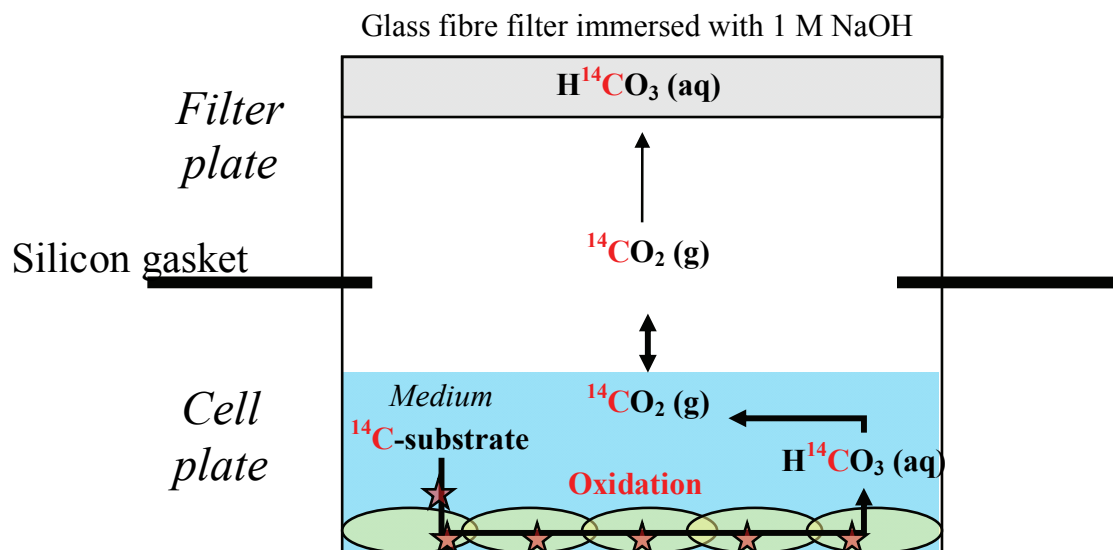
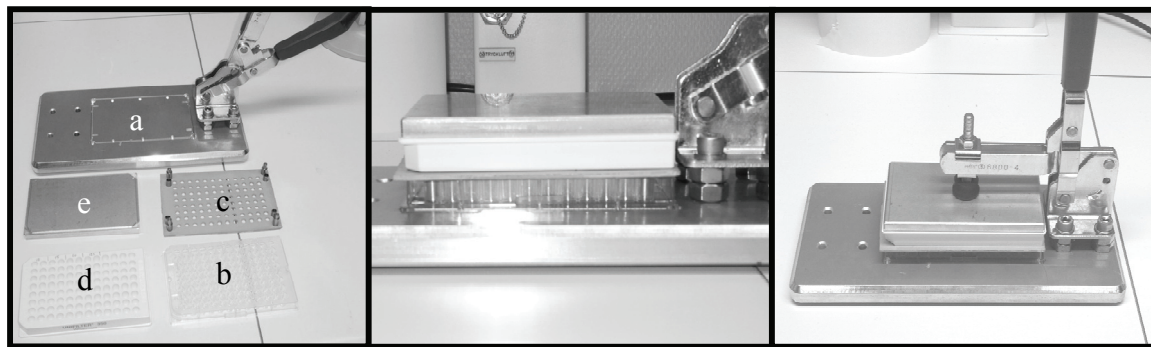


Figure 2.1 Equipment for CO₂ trapping and the principle behind the method.

2.5.4 Measurement of protein

1 part of the Bio-Rad protein dye was diluted with 4 parts of milliQ water. A volume of 50 μ l from each well of the cell lysate was transferred to a new 96 wells plate. 50 μ l 0.05 M NaOH was added to the last row of wells to assess bias. 200 μ l of the diluted protein reagents was added to each well. Bobbles were removed to avoid measurement interfere. Plates were let stand for 5 min and measured at 590 nm using a spectrophotometric microplate reader (Titertek Multiscan Plus (MK-II), LabSystems, Helsinki). The linear range of the assay for BSA is 0.2 to 0.9 mg/mL. For the purpose of quantification, a standard curve was used to calculate the amount of protein.

2.5.5 Cell-associated radiolabel (CAR)

A volume of 100 µl of cell lysate was removed from each well and transferred into a new opaque 96-well plate with transparent base plate. Scintillation fluid 200 µl was added. The plates were kept for 24 h in room temperature before measuring in the Microbeta trilux®.

2.5.6 Acid soluble metabolites (ASM)

A volume of 25 µl from the cell medium of each well was added to a 96-well plate with conic bottom. As a control, 25 µl of the medium used in the experiment was added to the last row. 25 µl BSA 6 % was added to each well. Liquids were well mixed by use of a pipette, before adding 250 µl cold perchloric acid (PCA) 1 M. The liquids were let stand 30-60 min at 4°C, and then spun down at 2800 rpm equaling 1480 G (Megafuge 1.0R) for 15 min at 4°C.

A volume of 100 µl of supernatant from each well was removed and transferred to an opaque 96-well plate. Scintillation fluid 200 µl was added. The plates were let standing for 24 h before measuring in the Microbeta trilux®.

2.5.7 Resolved acid soluble metabolites

The rest of the supernatant from plates used for ASM measurement was removed. 200 µl SDS-NaOH solution was added to each well. Plates were then covered with plastic and left at room temperature until resolved. Then 100µl was transferred to an opaque 96-well plate and 200 µl scintillation liquid added. The plates were let standing for approximately 24 h before measuring in the Microbeta trilux®.

2.6 Validation of the CO₂ trapping method

The CO₂ trapping method was verified with the use of Na¹⁴CO₃ counted with scintillation fluid (Ophtiphase supermix, Perkin Elmer, Shelton, CT, USA) in Microbeta trilux®. The aim was to find the most optimal conditions for measuring the actual activity in disintegrations per minute (DPM).

To verify the use of the Microbeta triluX® and the change in 96 well filter plates from “deep wells” (300 µl) to “shallow wells” (75 µl), the values of the actual activity in DPM from the multi-well experiment was compared with the efficacy of measurements in single tubes. The effect of pH in this experiment was tested out with diluting the radioactive solution added to the wet wells from 1 M to 0.1 M NaOH. The dry wells were let to dry for 24 h on a warm plate. Then the scintillation fluid was added and the plates were counted. The plates were counted every day for 5 days to see if there is a difference in counting efficacy if the plates are left for a while before counting (due to circulation).

6 ml 1 M NaOH was added 6.6 µl Na¹⁴CO₃ (1 mCi/mL, final concentration 1 µCi/mL). 25 µl of this solution was added to wells and to counting tubes. The solution was added to every second well to avoid contamination of the counting results in the neighboring wells. Both deep and shallow wells were counted when wet and one parallel of each after let to dry overnight.

The scintillation fluid was diluted with water in the ratios 1:0, 2:1, 1:1 and 2:3. 50 µl was added to the wet shallow wells, 200 µl was added to the wet deep wells, 75 µl was added to the shallow dry wells and 300 µl was added to the deep dry wells.

In a different experiment, isoplates with transparent base plate were used, and filters cut out and fitted in the wells. These filters were added the same amount of radioactive solution, but only 50 µl scintillation fluid and was counted on the cell lysat program (counted from both sides). Some of the wells without filters were added radioactive oleic acid (1 µCi/mL) and the same amount of scintillation fluid.

These results were compared with shallow filter plates, one with a transparent plastic cover and one with a white plastic covering the back of the plate.

(The results from these experiments are not shown).

2.7 Statistical analysis

All data from experiments performed more than three times were analyzed by paired t-test, assuming normal, two-tailed distribution of investigational variables. Average values for

each measurement were added standard error of the mean for experiments performed less than three times.

3. Results

3.1 Incubation of HepG2 cells with fatty acids

The natural fatty acids (FAs) tested have fasting physiological plasma concentrations between 10 and 400 μM (21). We therefore incubated HepG2 cells with either 50, 100 or 200 μM of the different natural FAs as well as TTA, TTTA and αTTA , to assess potential concentration effect on AR1 and AR2 mRNA expression. Results are shown in figures 3.1-4. Saturated fatty acids are known to induce insulin resistance more than unsaturated fatty acids. The physiological concentrations used for the saturated FA were also chosen for the Thia-FA so the effects on AR expression could be compared.

Adiponectin receptor 1 (AR1) mRNA related to fatty acids

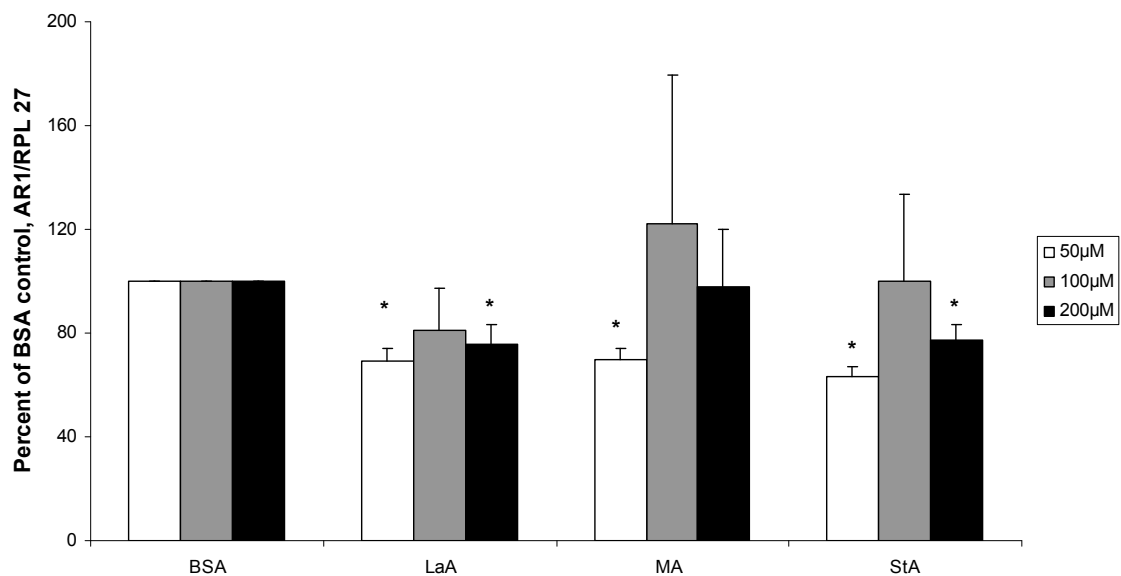


Figure 3.1 HepG2 cells incubated with fatty acids for 24 h before analyzed for content of adiponectin receptor 1 mRNA by RT-PCR. The fatty acids used were bovine serum albumin (BSA, essentially FA free) as a control, lauric acid (LaA, 12:0), myristic acid (MA, 14:0) and stearic acid (StA, 18:0) in 50 μM , 100 μM and 200 μM concentrations. Values are normalized with BSA as 100 %. Values are the mean of four independent experiments presented as mean \pm S.E.M (bars), $n = 4$. Asterisk (*) indicates significant changes ($P \leq 0.05$, paired t-test)

Expression of AR1 in HepG2 cells was significantly decreased after pre-incubation with LaA (50, 200 μ M) ($P=0.008$ and 0.048 respectively), MA (50 μ M) ($P=0.006$) and StA (50, 200 μ M) ($P=0.003$ and 0.034 , respectively).

Adiponectin receptor 1 (AR1) mRNA related to fatty acids

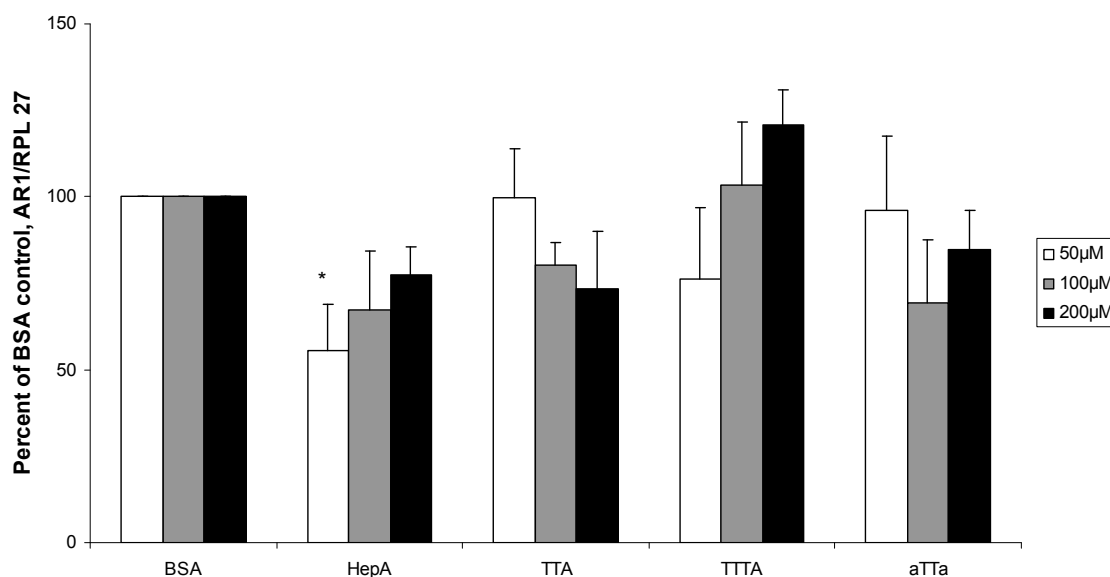


Figure 3.2 HepG2 cells incubated with fatty acids for 24 h before analyzed for content of adiponectin receptor 1 mRNA by RT-PCR. The fatty acids used were bovine serum albumin (BSA, essentially FA free) as a control, heptadecanoic acid (HepA, 17:0), tetradecylthioacetic acid (TTA, C2S14:0), triple TTA (TTTA) and alphasamethylTTA (α TTA) in 50 μ M, 100 μ M and 200 μ M concentrations. The values were normalized with BSA as 100 %. Values are the mean of four independent experiments presented as mean \pm S.E.M (bars), $n = 4$. Asterisk (*) indicates significant changes ($P \leq 0.05$, paired t-test).

Figure 3.2 shows that expression of AR1 mRNA in HepG2 cells was significantly decreased after pre-incubation with HepA (50 μ M) ($P=0.045$).

The FA analogs TTA, TTTA and α TTA in concentration 50, 100 and 200 μ M showed no significant reduction in adiponectin receptor 1 mRNA in these experiments.

Adiponectin receptor 2 (AR2) mRNA related to fatty acids

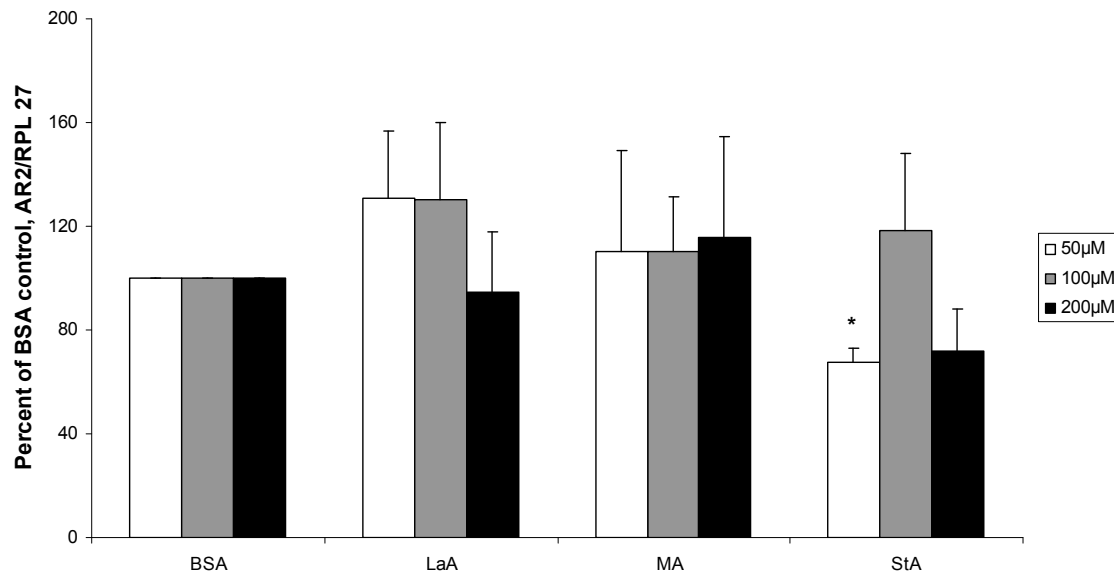


Figure 3.3 HepG2 cells were incubated with fatty acids for 24 h before analyzed for expression of adiponectin receptor 2 mRNA by RT-PCR. The fatty acids used were bovine serum albumin (BSA, essentially FA free) as a control, lauric acid (LaA, 12:0), myristic acid (MA, 14:0) and stearic acid (StA, 18:0) in 50 μ M, 100 μ M and 200 μ M concentrations. Values are normalized with BSA as 100 %. Values are the mean of four independent experiments presented as mean \pm S.E.M (bars), n = 4. Asterisk (*) indicates significant changes ($P \leq 0.05$, paired t-test).

Figure 3.3 shows that expression of AR2 mRNA in HepG2 cells was significantly decreased after pre-incubation with StA (50 μ M) ($P=0.008$). Other FAs showed no significant changes in mRNA expression compared to BSA control.

Adiponectin receptor 2 (AR2) mRNA related to fatty acids

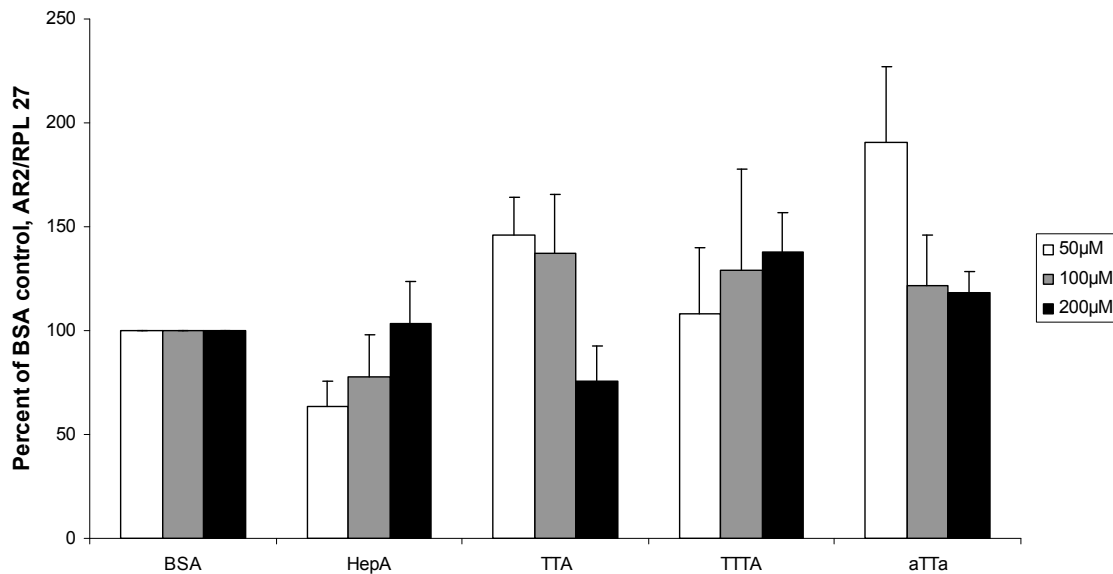


Figure 3.4 HepG2 cells incubated with fatty acids for 24 h before analyzed for expression of adiponectin receptor 2 mRNA by RT-PCR. The fatty acids used were bovine serum albumin (BSA, essentially FA free) as a control, heptadecanoic acid (HepA, 17:0), tetradecylthioacetic acid (TTA, C2S14:0), triple TTA (TTTA) and alphasamethylTTA (α TTA) in 50 μ M, 100 μ M and 200 μ M concentrations. The values were normalized with BSA as 100 %. Values are the mean of four independent experiments presented as mean \pm S.E.M (bars), n = 4. Asterisk (*) indicates significant changes ($P \leq 0.05$, paired t-test).

Figure 3.4 shows that no significant reduction in adiponectin receptor 2 mRNA was observed in experiments with HepA and TTA, TTTA and α TTA FA analogs in concentrations 50, 100 and 200 μ M.

3.1.1 Lactate dehydrogenase (LDH)-activity in cell culture media

For some of the FA incubations, the HepG2 cells changed appearance when they were investigated in the microscope compared to the cells incubated with BSA, where the cells kept their natural appearance. Cells clustered together and did not stretch over the surface in the same way as for BSA incubated cells. The LDH was measured in the media after 24 h of pre-incubation with FAs, and related to total LDH amount after complete cell lysis.

Lactate dehydrogenase (LDH) leakage related to fatty acids

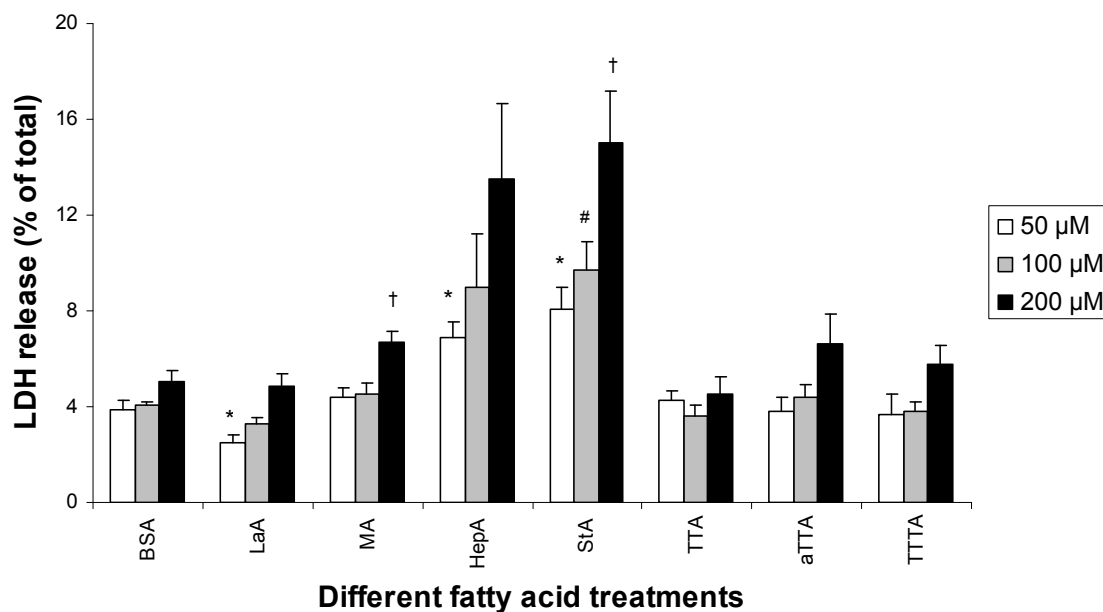


Figure 3.5 Leakage of LDH into media as percent of total release after incubating HepG2 cells with fatty acids. The fatty acids used were bovine serum albumin (BSA, essentially FA free) as a control, lauric acid (LaA, 12:0), myristic acid (MA, 14:0), heptadecanoic acid (HepA, 17:0), stearic acid (StA, 18:0), tetradecylthioacetic acid (TTA, C2S14:0), alphasamethylTTA (α TTA) and triple TTA (TTTA) in 50 μ M, 100 μ M and 200 μ M concentrations. Values are the mean of four independent experiments mean percentage \pm S.E.M (bars), $n = 4$. * indicates significant changes in LDH values between 50 μ M BSA and other FAs present at 50 μ M, # indicates significant changes in LDH values between BSA 100 μ M and other FAs present at 100 μ M and † indicates significant changes in LDH values between 200 μ M BSA and other FAs present at 200 μ M, respectively ($P \leq 0.05$, paired t-test).

Figure 3.5 shows that the long saturated FAs, HepA and StA, increases LDH release. More specifically, significant changes were found when comparing BSA 50 μ M with; StA 50 μ M

($P=0.049$), LaA 50 μM ($P=0.007$), HepA 50 μM ($P=0.040$), BSA 100 μM with StA 100 μM ($P=0.017$) and BSA 200 μM with; MA 200 μM ($P=0.029$) and StA 200 μM ($P=0.019$). For all other FAs, there were no significant changes in LDH release.

Significant dose dependent LDH elevations with increasing FA concentrations were also found when comparing different concentrations of the same FA. More specifically, there was a FA concentration-dependent LDH increase from MA 50 μM to MA 200 μM ($P=0.005$), from MA 100 μM to MA 200 μM ($P=0.029$), from StA 50 μM to StA 200 μM ($P=0.037$), from StA 100 μM to StA 200 μM ($P=0.029$), from LaA 50 μM to LaA 100 μM ($P=0.011$), from LaA 50 μM to LaA 200 μM ($P=0.007$), from LaA 100 μM to LaA 200 μM ($P=0.012$), from HepA 100 μM to HepA 200 μM ($P=0.047$) and from aTTA 50 μM to aTTA 100 μM ($P=0.006$).

3.2 Quantification of T-cadherin mRNA

3.2.1 Primer design and testing

Primers suited for qRT-PCR (product length < 280 bp) targeting the human T-cadherin coding region were designed using NCBI Sequence Viewer and the sequence CDS 121-2262 bp (gi: 61676095, gi for CDS: 4502719). The primer pair was initially tested in a Light Cycler instrument and cDNA from HepG2 cells, synthesized with reverse transcriptase from isolated total RNA, was used as a template. The PCR product was analyzed by agarose gel (1%) electrophoresis and several unspecific bands appeared. To make the reaction conditions optimal, several parameters were changed. Different annealing temperatures (55°C - 65°C), annealing times (3-5 sec.) and MgCl_2 concentrations (1.6–3.2 μl) were tested without improving the specificity of the reaction.

Therefore, a new set of primers was designed and tested. Still unspecific bands showed up when the PCR product was analyzed on an agarose gel (1%). Then, cDNA was tested from human heart tissue. The PCR reaction with human heart tissue cDNA template produced a single 280 bp band on an agarose gel, as showed in figure 3.6. Water was used as a negative control in all the tests.

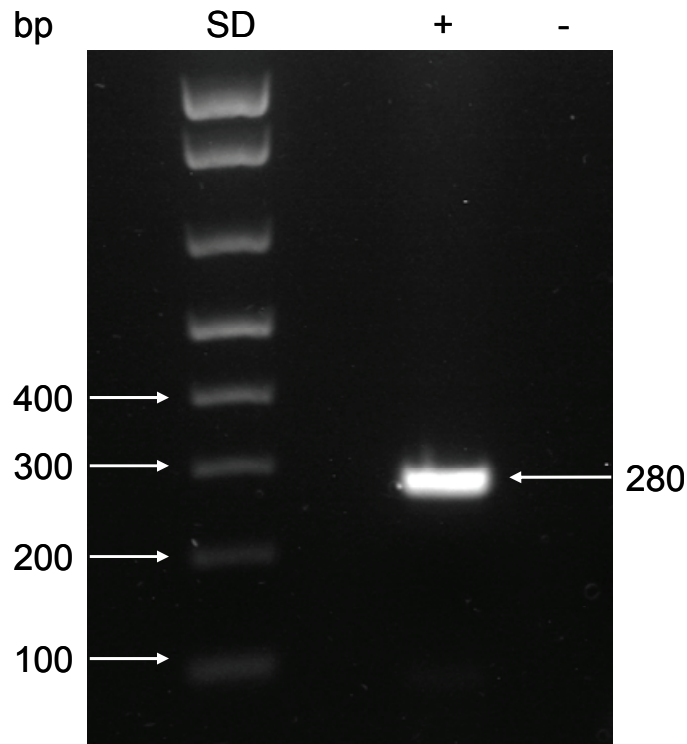


Figure 3.6 Testing of candidate primer pairs for use in qRT-PCR. PCR product of T-cadherin (280 bp) and negative control (water) were run on agarose gel (1 %). SD = 1 kb Plus ladder

3.2.2 Cloning of the protein encoding region of T-cadherin for use as a positive control and standard curve in qRT-PCR

In the second step of establishing a qRT-PCR method for the measurement of T-cadherin, the complete protein coding region for this gene was cloned. Verified DNA (cut out from plasmid DNA by restriction enzymes) from the clone was used as positive control and serial diluted standard curves in qRT-PCR with the primer pair hTCAD-R and hTCAD-F. Blunt end-products were cloned into the pENTR/D-TOPO vector and transformed into *E. coli* bacteria.

The plasmids were added restriction enzymes (Sal I and Not I enzymes) to analyze and identify which plasmid contained the complete protein coding region inserted in the correct direction.

After analysis through agarose gel electrophoresis, one clone of a total of 10 clones was identified yielding the predicted bands 1652 bp (1636 insert and 16 bp) and all vector (2580

bp and (501 bp-16 bp)) 3069 bp (sample number 3 figure 3.7). This restriction enzyme analysis was performed as a control of both the size of the PCR-product and to exclude confusion with super-coiled plasmid DNA.

The complete coding region inserted in the cloned plasmid was control sequenced twice by MedProbe (Norway), and the resulting sequence was verified by alignment (BLAST) with the original GENE BANK mRNA sequences.

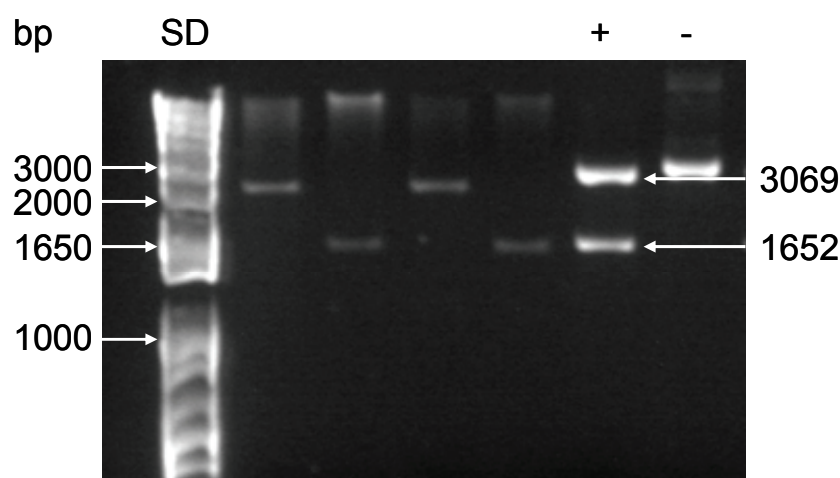


Figure 3.7 Cloning of complete coding region of T-cadherin receptor, for use as a control and standard in qRT-PCR. Plasmid DNA (Miniprep) incubated with the Not I and Sal I restriction enzymes and then analyzed by agarose gel electrophoresis (1 %). SD = 1 kb Plus ladder

3.3 Expression of T-cadherin receptor

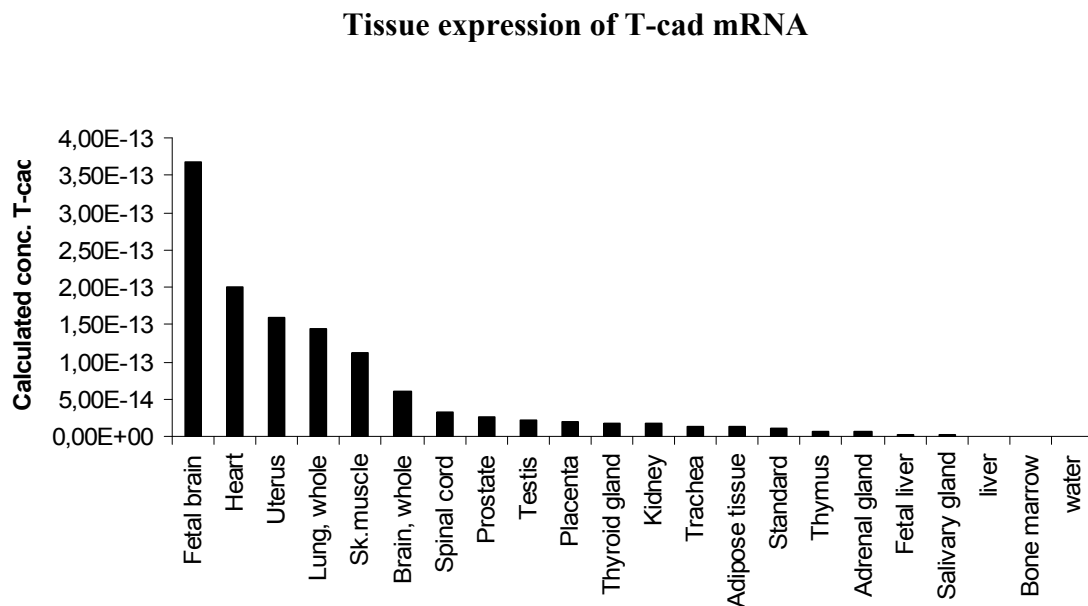


Figure 3.8. Expression of T-cadherin mRNA in a tissue panel. mRNA values were measured by RT-PCR and normalized by use of RPL27 as an internal control. The calculated values were measured with the use of a standard curve for T-cadherin receptor (n=2).

Figure 3.8 shows that the distribution of T-cadherin receptor mRNA in the body varies substantially, where fetal brain exhibited highest expression, brain (whole) and spinal cord were moderately expressed and liver and bone marrow were barely detectable. Fetal brain contained a 1000-fold more T-cadherin than liver.

Expression of T-cad in different cell types

cDNA from a selection of cells was also analyzed for expression of T-cad receptor. cDNA from U937 control (monocytes) was first used to generate T-cadherin receptor DNA for the cloning process. cDNA was then analyzed by gel electrophoresis, and small amounts of T-cad in these cells were found. Other cells analyzed were SGBS cells undifferentiated and differentiated (day 1 and day 19) (stromal cell fraction of subcutaneous adipose tissue of an infant with Simpson Golabi Behmel syndrome), cDNA from experiments performed by

Andreas J Wensaas with cultured and differentiated primary human muscle cells (myotubes), and cDNA from BeWo (placental trophoblast cell-line), THLE (liver cell-line), Raji and Ramos (B cell-lines), Jurkat (T cell-line), THP-1 (monocyte cell line), PMA treated THP-1 (differentiated into macrophages)) and U937 (monocyte cell line), PMA treated U937 (macrophages).

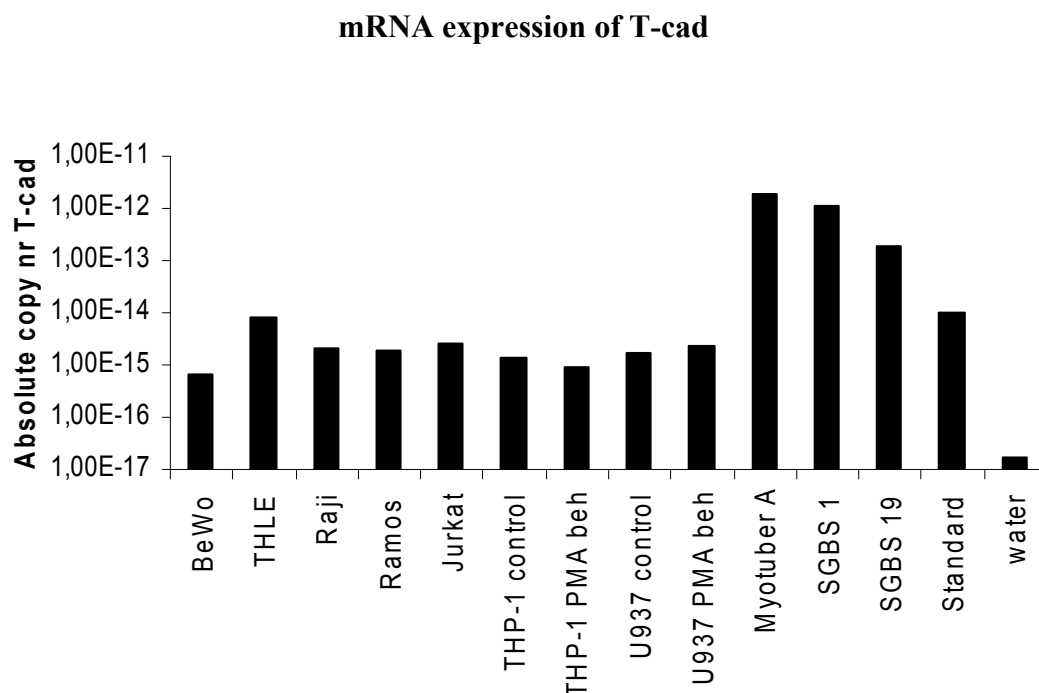


Figure 3.9 Expression of T-cadherin receptor mRNA in cells and selected malign lymphoid cell lines. The cDNA was analyzed by RT-PCR and quantified by use of a standard curve. The measured amount of T-cadherin mRNA is the average of two measurements (n=2).

Figure 3.9 shows that the amounts of T-cadherin receptor mRNA in different cells varied greatly. Myotubes and adipose tissue (SGBS cells) expressed 1000-fold more T-cadherin receptor mRNA compared to Raji, Ramos and Jurkat.

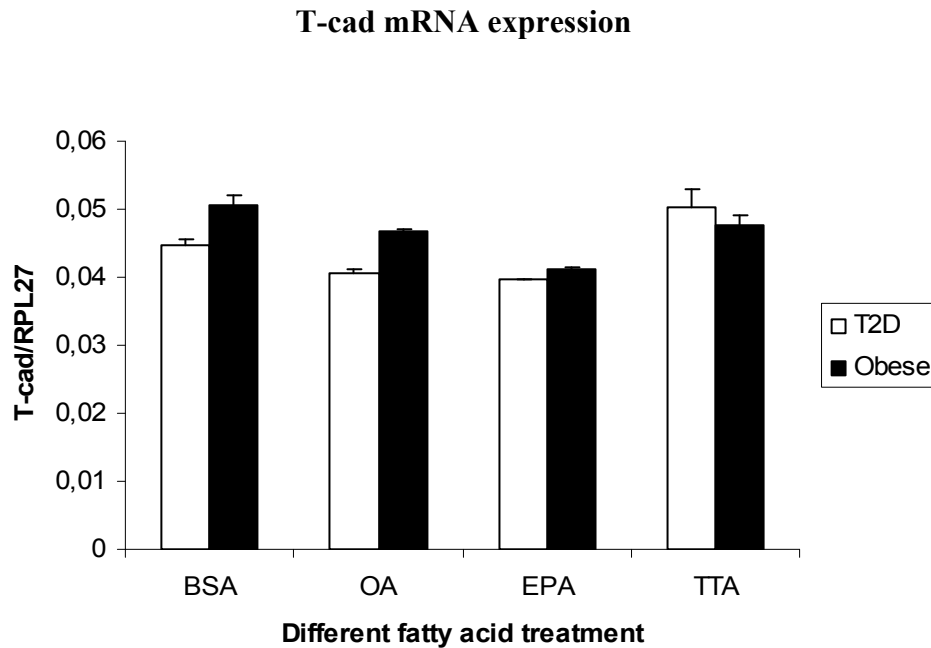


Figure 3.10 Expression of T-cadherin receptor mRNA in myotubes from 8 obese and 8 type 2-diabetic donors. The expression of T-cad was analyzed by RT-PCR and all measurements were normalized for housekeeping gene RPL27. The myotubes were incubated with bovine serum albumin (BSA) as a control, oleic acid (OA, (18:1, n-9)), eicosapentaenic acid (EPA, (20:5, n-3)) and tetradecylthioacetic acid (TTA (17:0, 3-thia)) at 100 μ M concentration. (n=2)

Figure 3.10 shows that oleic acid and eicosapentaenoic acid may decrease expression of T-cad by 9 and 11% respectively, compared to BSA in myotubes derived from type 2-diabetic donors. The apparent effect tetradecylthioacetic acid was an increase of 13 % compared to BSA. Oleic acid, eicosapentaenic acid and tetradecylthioacetic acid apparently decreased expression of T-cad by 7, 18 and 5 % respectively compared to BSA in myotubes derived from obese.

AR1 mRNA expression

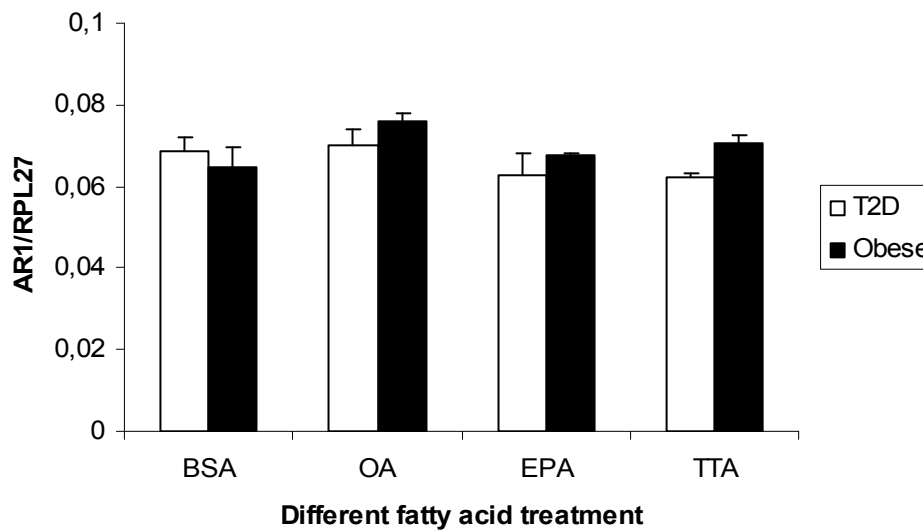


Figure 3.11 Expression of AR1 mRNA in myotubes from 8 obese and 8 type 2 diabetic donors. The expression of AR1 was analyzed by RT-PCR and all measurements were normalized for housekeeping gene RPL27. The myotubes were incubated with bovine serum albumin (BSA) as a control, oleic acid (OA, (18:1, n-9)), eicosapentaenic acid (EPA, (20:5, n-3)) and tetradecylthioacetic acid (TTA (17:0, 3-thia)) at 100 μ M concentration. (n=2)

Figure 3.11 shows that eicosapentaenic acid and tetradecylthioacetic acid apparently decreased the expression of AR1 by 9 and 10 % respectively compared to BSA in myotubes derived from type 2-diabetic donors.

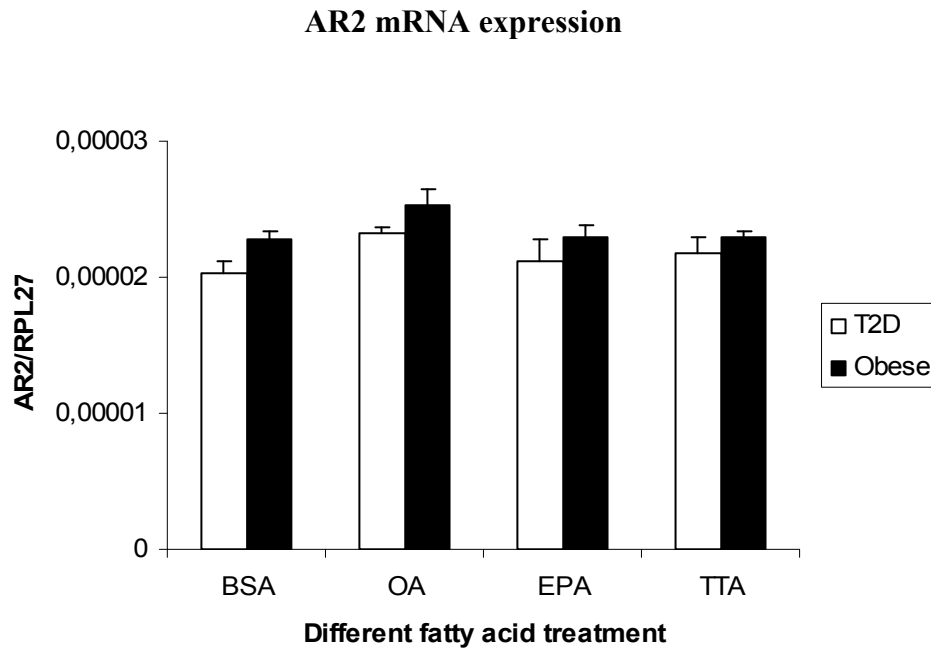


Figure 3.12 Expression of AR2 mRNA in myotubes from 8 obese and 8 type 2 diabetic donors. The expression of AR2 was analyzed by RT-PCR and all measurements were normalized for housekeeping gene RPL27. The myotubes were incubated with bovine serum albumin (BSA) as a control, oleic acid (OA, (18:1, n-9)), eicosapentaenic acid (EPA, (20:5, n-3)) and tetradecylthioacetic acid (TTA (17:0, 3-thia)) at 100 μ M concentration. (n=2)

Figure 3.12 shows that oleic acid, eicosapentaenic acid and tetradecylthioacetic acid apparently do not affect AR2 expression compared to BSA in myotubes derived from type 2-diabetic or obese donors.

3.4 Effect of adiponectin on human myotubes

3.4.1 Experiments with full-length adiponectin

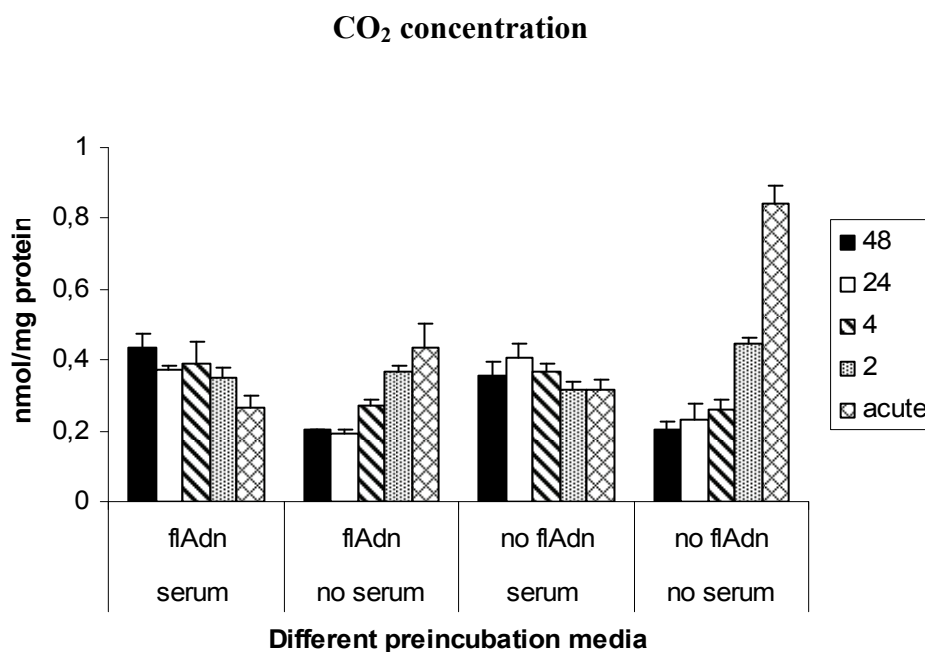


Figure 3.13 Effect of full-length adiponectin on CO₂ production after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were preincubated in media with or without adiponectin (0.5 µg/mL) and serum (2 %) for 2, 4, 24 or 48 h. Afterwards the myotubes were incubated for 4 h with [¹⁴C]oleic acid (1 µCi/mL 50 µM), L-carnitine (1 mM) and glucose (5 mM). The media added to the acute incubation were added adiponectin during the 4 hour incubation period. Radiolabeled CO₂ was trapped and quantified. The means ± standard deviation is given as absolute quantities of the parallels (n=4).

Figure 3.13 show that there is a possible decrease in oleic acid oxidation for the longest pre-incubation periods for cells grown in serum and adiponectin deficient medium, compared to acute incubation. Measured oleic acid oxidation seemed to decrease after extensive pre-incubation periods for cells pre-incubated with adiponectin containing medium deficit of serum. There seemed to be no increased effect of adiponectin related to FA oxidation on cells incubated with adiponectin and serum containing media, Pre-incubating cells with

medium containing serum and no adiponectin, seemed to have minor effect on oleic acid oxidation.

Acid Soluble Metabolites (ASM) concentration

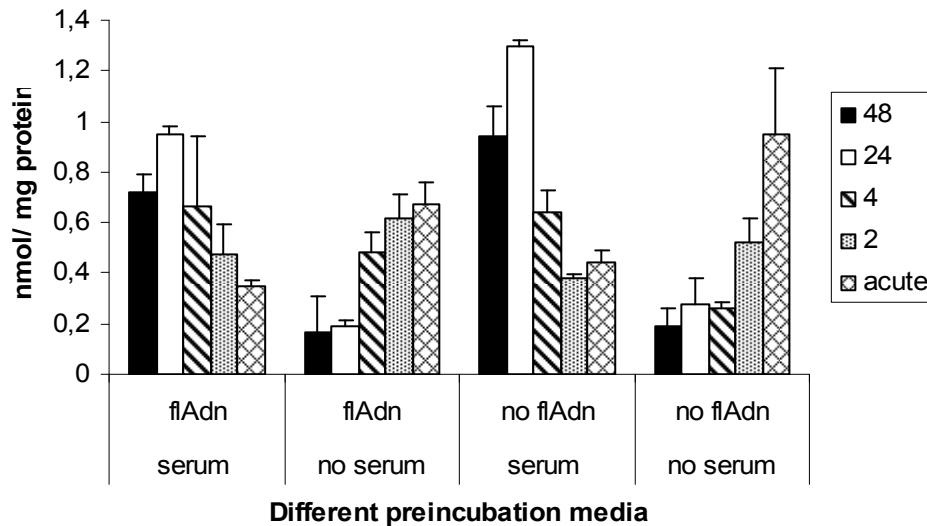


Figure 3.14 Effect of full-length adiponectin and serum on cell mediated ASM after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were preincubated in media with or without adiponectin (0.5 μ g/mL) and serum (2 %) for 2, 4, 24 or 48 h. Afterwards the myotubes were incubated for 4 h with [14 C]oleic acid (1 μ Ci/mL 50 μ M), L-carnitine (1 mM) and glucose (5 mM). The media added to the acute incubation were added adiponectin during the 4 hour incubation period. The means \pm standard deviation is given as absolute quantities of the 4 parallels of each incubation.

Figure 3.14 shows that cells pre-incubated with medium containing both serum and adiponectin seemed to increase oleic acid oxidation. The same tendency was seen for cells pre-incubated with medium containing only serum. For cells pre-incubated with medium containing adiponectin only and cells pre-incubated with plain medium, there seemed to be a tendency for increased oleic acid oxidation with shorter pre-incubation periods.

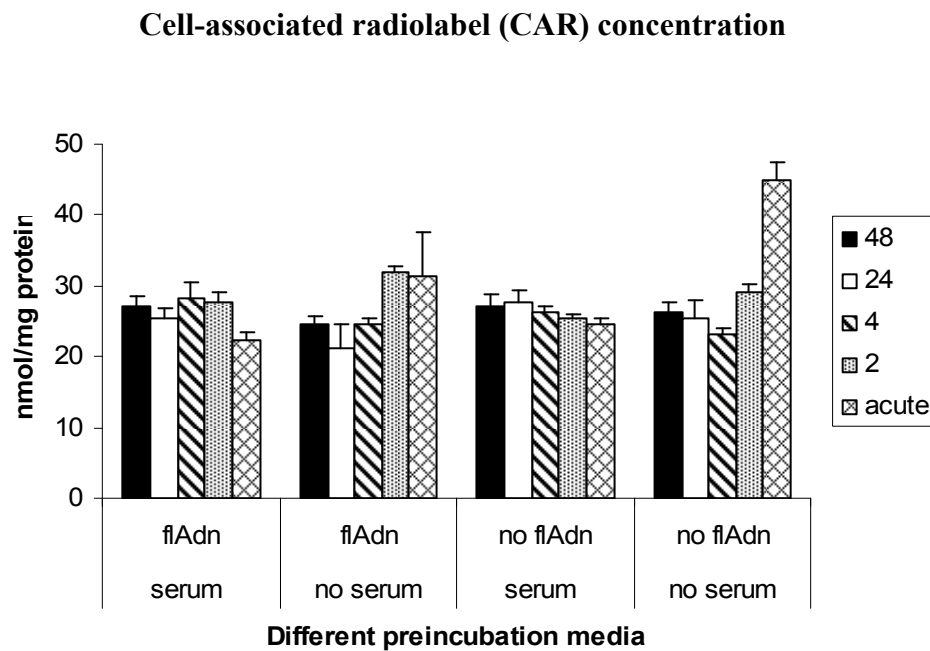


Figure 3.15 Effect of full-length adiponectin and serum on cell-associated radiolabel (CAR) after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were preincubated in media with or without adiponectin (0.5 µg/mL) and serum (2 %) for 2, 4, 24 or 48 h. Afterwards the myotubes were incubated for 4 h with [14 C]oleic acid (1 µCi/mL 50 µM), L-carnitine (1 mM) and glucose (5 mM). The media added to the acute incubation were added adiponectin during the 4 hour incubation period. The means \pm standard deviation is given as absolute quantities of the 4 parallels of each incubation.

Figure 3.15 shows no clear tendency towards an effect on cell-associated radiolabel measurements when adiponectin is added to the pre-incubation or incubation media. Regardless of adiponectin content, cells incubated with serum deficient medium seemed to increase cell-associated radiolabel measurements after a 2-hour pre-incubation period and acute incubation with adiponectin.

3.4.2 Experiments with globular adiponectin

Experiments were performed with four different pre-incubation time intervals and different concentrations of adiponectin (figure 3.16 to 3.21). In the first experiments myotubes were pre-incubated with gAdn for 72, 48, and 24 h or had adiponectin added to the media only during the 4 hour incubation with oleic acid. In the second experiment with gAdn myotubes were pre-incubated for 24, 4 and 2 h or had adiponectin added to the media only during the 4 hour incubation with oleic acid.

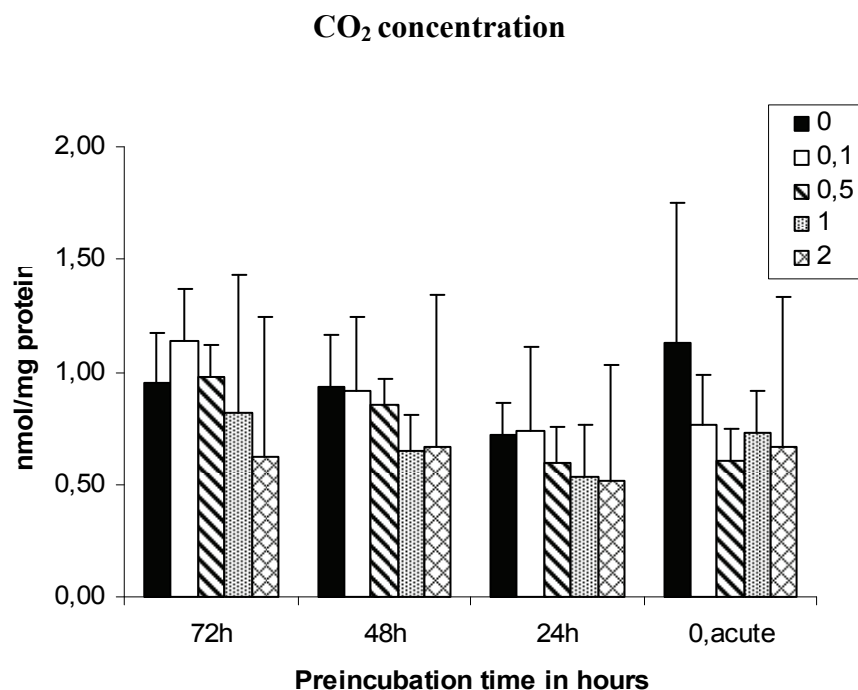


Figure 3.16 Effect of globular adiponectin on CO₂ measurements after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were added media containing various amounts of adiponectin at defined time intervals. The experiment was performed by preincubating the cells for 24-, 48- or 72 h with different media and incubating the cells for 4 h with [1-¹⁴C]oleic acid (1 µCi/mL, 50 µM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). The adiponectin concentrations used were 0.1, 0.5, 1 and 2 µg/mL. The media for the acute incubation were added adiponectin during the 4 hour incubation period. Radiolabeled CO₂ was trapped and quantified. The means ± SEM is given as absolute quantities of the 8 parallels.

For cells pre-incubated with the lowest adiponectin concentrations there seemed to be a tendency for an increase in oleic acid oxidation with increasing pre-incubation time (figure

3.16). There seemed to be a tendency for a decreased OA oxidation when pre-incubating cells in higher concentrations of adiponectin. However, none of the measured changes in CO₂ were statistically significant.

Acid Soluble Metabolites (ASM) concentration

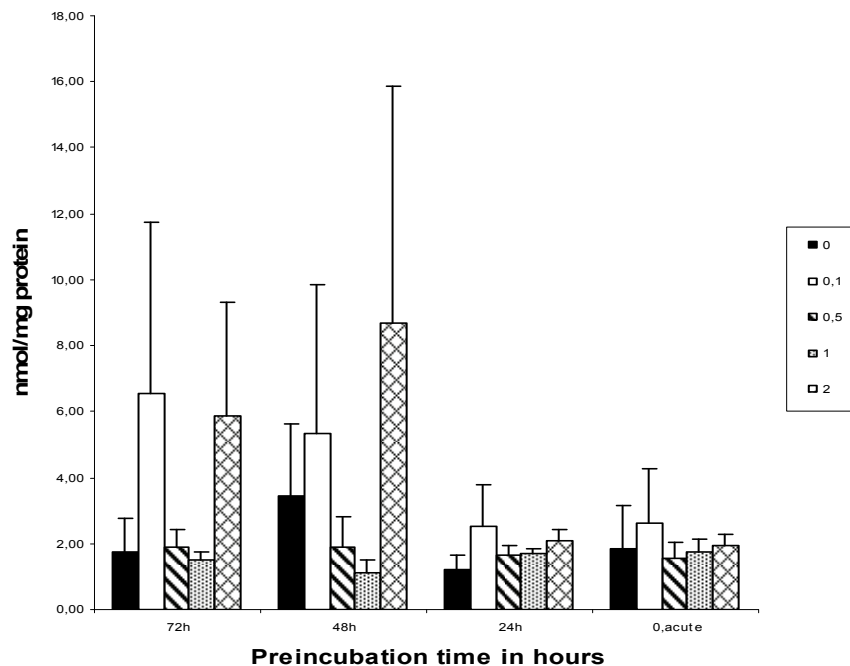


Figure 3.17 Effect of globular adiponectin on acid soluble metabolites (ASM) after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were added media containing various amounts of adiponectin at defined time intervals. The experiment was performed by pre-incubating the cells for 24, 48 or 72 h with different media and incubating the cells for 4 h with [$1\text{-}^{14}\text{C}$]oleic acid (1 $\mu\text{Ci/mL}$, 50 μM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). The adiponectin concentrations used were 0.1, 0.5, 1 and 2 $\mu\text{g/mL}$. The media added to the acute incubation were added adiponectin during the 4 hour incubation period. The means \pm SEM is given as absolute quantities of the 8 parallels.

Figure 3.17 shows that the standard deviations for some of the ASM measurements are relatively high and therefore no conclusions regarding an effect of gAdn can be drawn based on these results.

The next experiment that was performed with globular adiponectin, shorter time intervals were chosen; 24 h, 4 h, 2 h and acute incubation with adiponectin. Lower doses of adiponectin were used; 0, 0.1, 0.25, 0.5 and 1 $\mu\text{g/mL}$.

Cell-associated radiolabel (CAR) concentration

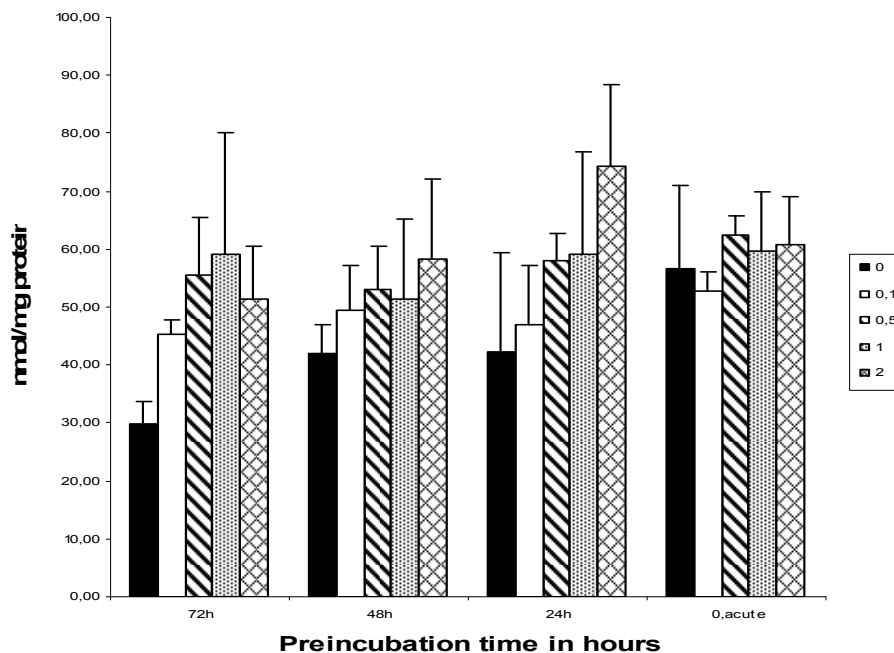


Figure 3.18 Effect of globular adiponectin on cell-associated radiolabel (CAR) measurement after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were added media containing various amounts of adiponectin at defined time intervals. The experiment was performed by preincubating the cells for 24-, 48- or 72 h with different media and incubating the cells for 4 h with [^{14}C]oleic acid (1 $\mu\text{Ci/mL}$, 50 μM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). The adiponectin concentrations used were 0.1, 0.5, 1 and 2 $\mu\text{g/mL}$. The media for the acute incubation were added adiponectin during the 4 hour incubation period. The means \pm S.E.M. is given as absolute quantities of the 8 parallels.

For the cell-associated radiolabel (CAR) measurements there was a tendency towards an increased effect for the highest adiponectin concentrations used (figure 3.18). There seemed to be no increase in CAR measurements between the 72h- 48h- and the 24 hour pre-incubation times. No changes in the measured CAR values were statistically significant.

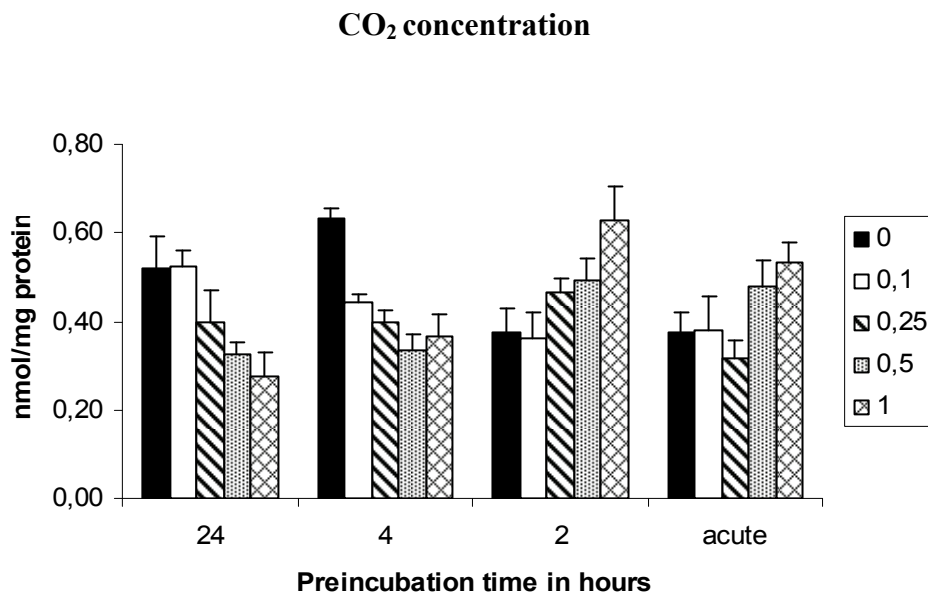


Figure 3.19 Effect of globular adiponectin on CO₂ measurements after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were added media containing various amounts of adiponectin at defined time intervals. The experiment was performed by preincubating the cells for 2-, 4- or 24 h with different media and incubating the cells for 4 h with [1-¹⁴C]oleic acid (1 $\mu\text{Ci/mL}$, 50 μM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). The adiponectin concentrations used were 0.1, 0.25, 0.5 and 1 $\mu\text{g/mL}$. The media added to the acute incubation were added adiponectin during the 4 hour incubation period. Radiolabeled CO₂ was trapped and quantified. The means \pm S.E.M. is given as absolute quantities of the 8 parallels.

Cells pre-incubated with adiponectin for 24 and 4 h showed a decrease in oleic acid oxidation with increasing concentration of adiponectin (figure 3.19). Adiponectin seemed to have a stimulating effect on the amount of fatty acid oxidation for the cells pre-incubated for 2 h and acute incubation with adiponectin during the 4 hour incubation time.

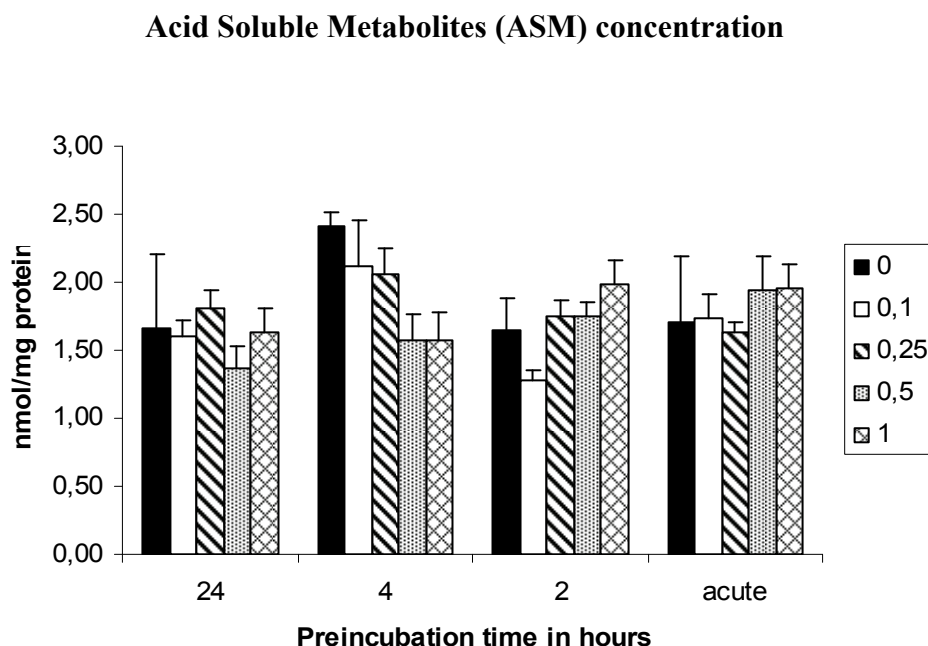


Figure 3.20 Effect of globular adiponectin on acid soluble metabolites (ASM) after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were pre-incubated in media containing 0, 0.1, 0.25, 0.5 and 1 µg/mL adiponectin and pre-incubated for 2, 4 or 24 h. The myotubes were later incubated for 4 h with [14 C]oleic acid (1 µCi/mL, 50 µM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). For the acute incubation, adiponectin was added to the medium during the 4 hour incubation period. The means \pm SEM is given as absolute quantities of 8 parallels.

There was no tendency for an effect of pre-incubating myotubes for 24 h with increasing concentration of gAdn, as shown in figure 3.20. For the 4 hour pre-incubation time a tendency for a decrease in measured ASM values with increasing concentration of gAdn was seen. For the 2 hour pre-incubation and acute incubation with gAdn no tendency towards an increase in measured ASM values was observed that was related to the concentration of gAdn.

Cell-associated radiolabel (CAR) concentration

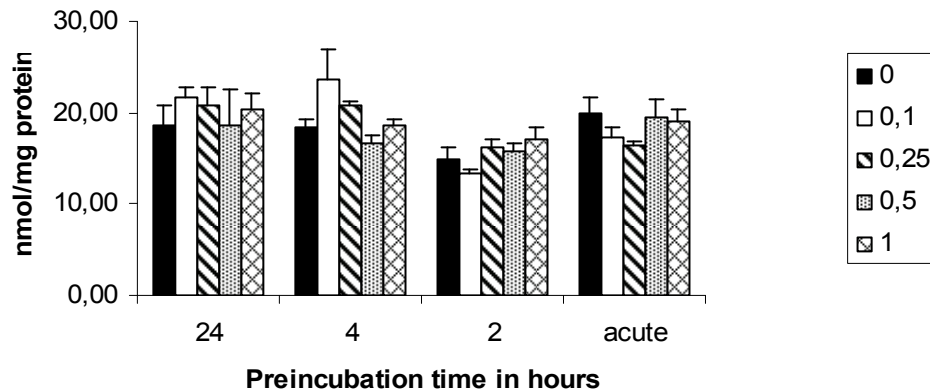


Figure 3.21 Effect of globular adiponectin on cell-associated radiolabel (CAR) after incubation with radioactive oleic acid for 4 h. Differentiated myotubes were added media containing various amounts of adiponectin at defined time intervals. The experiment was performed by preincubating the cells for 2-, 4- or 24 h with different media and incubating the cells for 4 h with [^{14}C]oleic acid (1 $\mu\text{Ci/mL}$, 50 μM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). The adiponectin concentrations used were 0.1, 0.25, 0.5 and 1 $\mu\text{g/mL}$. The media added to the acute incubation were added adiponectin during the 4 hour incubation period. The means \pm SEM is given as absolute quantities of the 8 parallels.

Measured values for the effect of gAdn on CAR measurements showed no clear tendencies towards an effect when comparing the pre-incubation times and concentrations of gAdn (figure 3.21).

Lactate Dehydrogenase (LDH) release

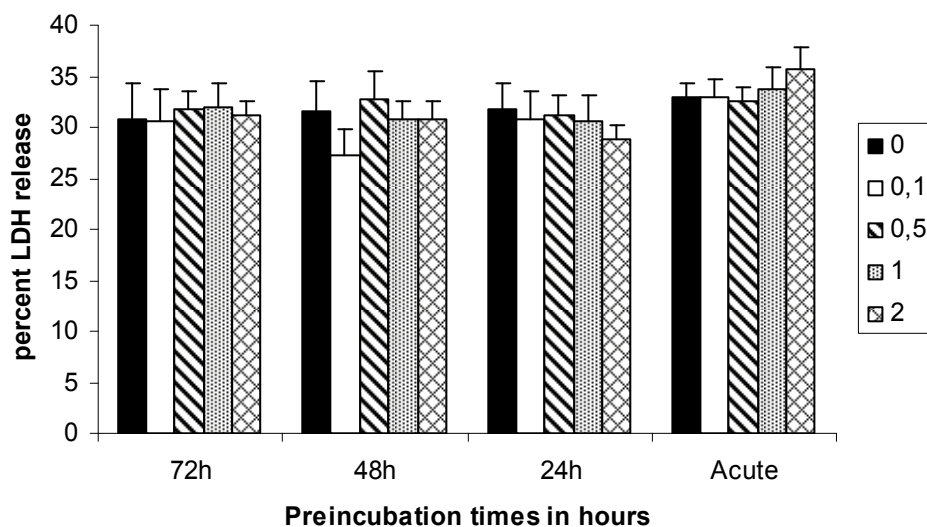


Figure 3.22 Measure of Lactate Dehydrogenase (LDH) values in cell media after 4 h incubation with [1-¹⁴C]oleic acid (50 µM, 1 µCi/mL). Differentiated myotubes were added media containing different amounts of adiponectin at different time intervals. The experiment was performed by preincubating the cells for 24-, 48- or 72 h with different media and incubating the cells for 4 h with [1-¹⁴C]oleic acid (1 µCi/mL, 50 µM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). The different adiponectin concentrations used were 0.1, 0.5, 1 and 2 µg/mL. The media added to the acute incubation were added adiponectin during the 4 hour incubation period. 25 µl of cell medium from each well were diluted with 25 µl water and added 50 µl LDH-kit reagents. The plate was incubated for 10 min at 37°C wrapped in aluminum foil. Amount LDH was measured with a spectrophotometer at 492 nm. The means ± S.E.M. is given as absolute quantities of the 4 parallels.

In figure 3.22 the lactate dehydrogenase (LDH) measurements show no significant changes in measured values for different pre-incubation times and gAdn concentrations.

3.4.3 Experiments with trimer adiponectin

Cells received trimer adiponectin at two different time intervals, 24 h and acute. Cells were then tested for oleic acid and glucose oxidation.

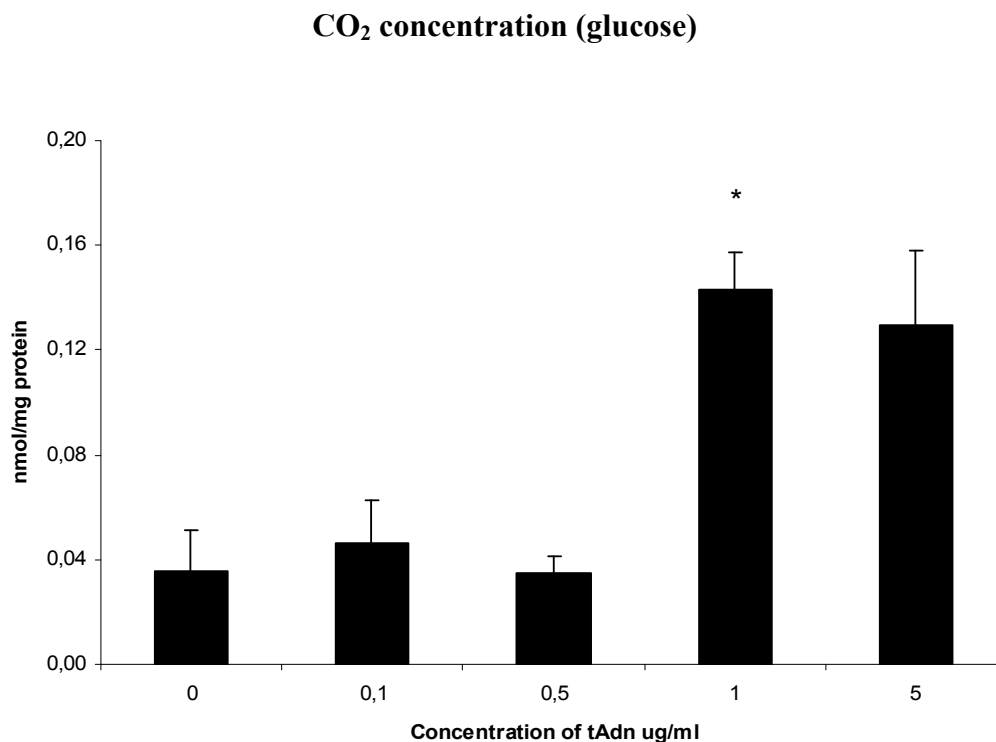


Figure 3.23 Effect of acute incubation with trimer adiponectin (tAdn) on glucose oxidation. Differentiated myotubes were incubated for 4 h with [6-¹⁴C]glucose medium (1 μ Ci/mL, 100 μ M) and 0.2 % Fetal Calf Serum (FCS). Trimer adiponectin concentrations in the medium were 0 (control), 0.1, 0.5, 1 and 5 μ g/mL. Radiolabeled CO₂ was trapped and quantified. The means \pm SEM is given as absolute quantities of 12 parallels (3 individual experiments with 4 parallels each). Asterisk (*) indicates significant change compared to control ($p \leq 0.05$, two-tailed t-test).

Trimer adiponectin significantly increased glucose oxidation (figure 3.23). A concentration of 1 μ g/mL tAdn caused a 4-fold increase in glucose oxidation compared to control incubated without tAdn ($P = 0.05$). A significant increase in CO₂ measurements was not found for the myotubes incubated with 5 μ g/mL tAdn.

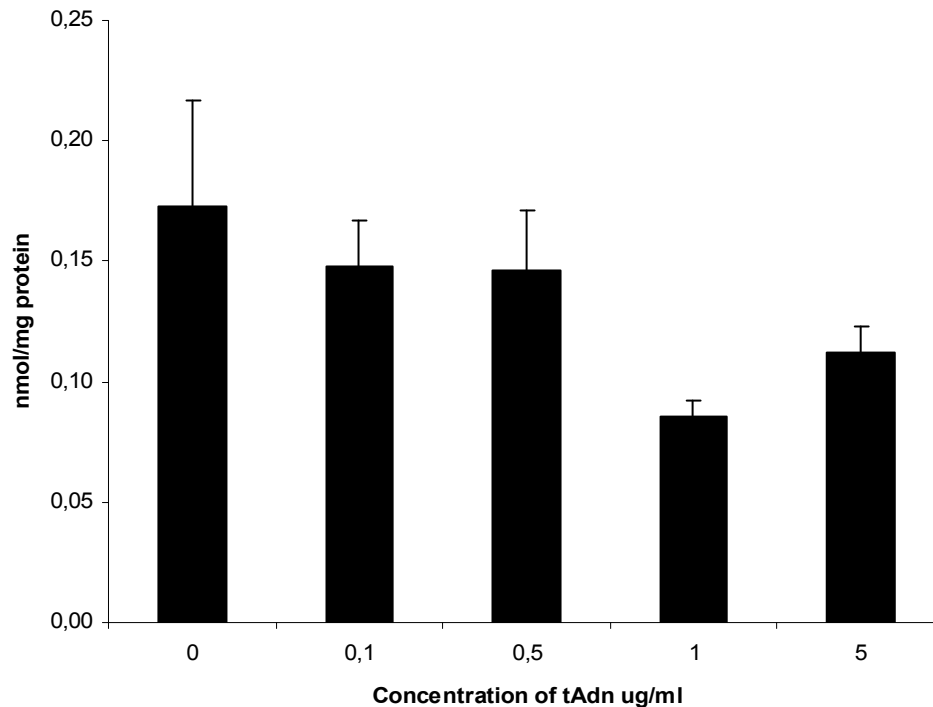
CO₂ concentration (glucose)

Figure 3.24 Effect of 24 h pre-incubation with trimer adiponectin (tAdn) on glucose oxidation. Differentiated myotubes were pre-incubated 24 h with medium containing 0 (control), 0.1, 0.5, 1 and 5 $\mu\text{g/mL}$ tAdn. The myotubes were then incubated for 4 h with $[6\text{-}^{14}\text{C}]$ glucose (1 $\mu\text{Ci/mL}$, 100 μM) and 0.2 % Fetal Calf Serum (FCS). Radiolabeled CO_2 was trapped and quantified. The means \pm SEM is given as absolute quantities of 12 parallels (3 individual experiments with 4 parallels each).

As seen in figure 3.24 there was a tendency for a decrease in glucose oxidation with increasing amounts of tAdn in the pre-incubation medium, but no significant changes was observed.

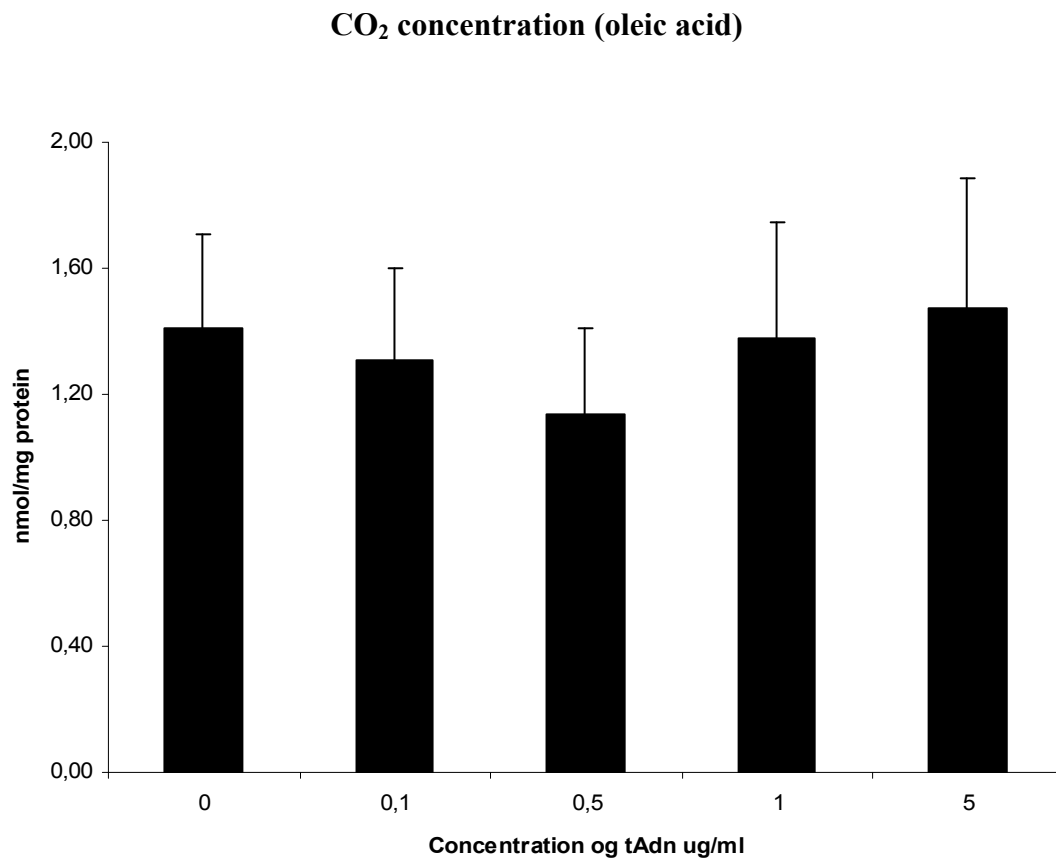


Figure 3.25 Effect of acute incubation with trimer adiponectin (tAdn) on oleic acid oxidation. Differentiated myotubes were incubated for 4 h with [1-¹⁴C]oleic acid (1 μ Ci/mL, 50 μ M), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). Medium tAdn concentrations were 0 (control), 0.1, 0.5, 1 and 5 μ g/mL. Radiolabeled CO₂ was trapped and quantified. The means \pm SEM are given for 12 parallels (3 individual experiments with 4 parallels each).

Figure 3.25 shows results of acute incubation with tAdn. There was no clear trend for a change in FA oxidation with increasing amounts of tAdn in the medium, compared to the myotubes incubated without tAdn.

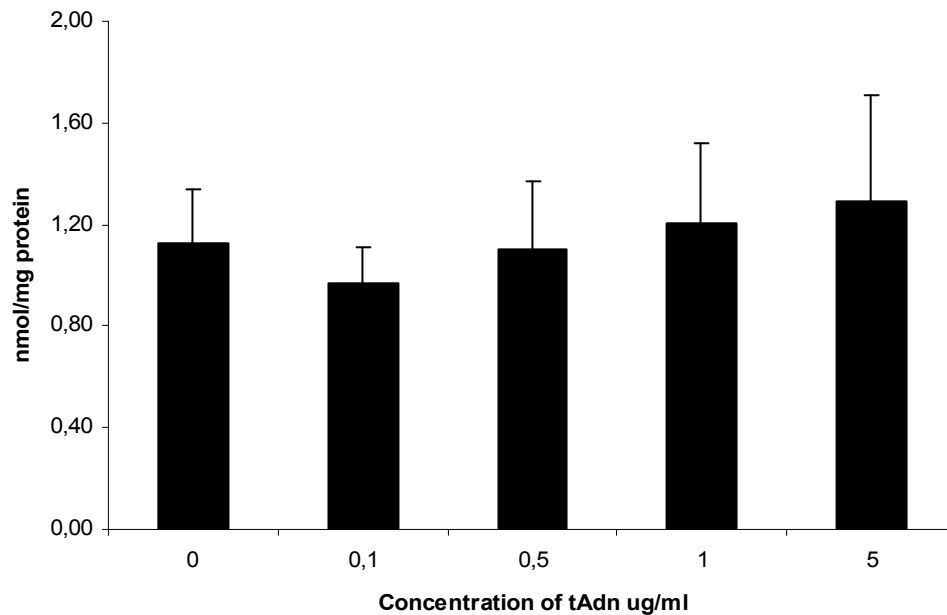
CO₂ concentration (oleic acid)

Figure 3.26 Effect of 24 h pre-incubation with trimer adiponectin (tAdn) on oleic acid oxidation. Differentiated myotubes were pre-incubated 24 h with medium containing 0 (control), 0.1, 0.5, 1 and 5 $\mu\text{g/mL}$ tAdn. The myotubes were then incubated for 4 h with $[1\text{-}^{14}\text{C}]$ oleic acid (1 $\mu\text{Ci/mL}$, 50 μM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). Radiolabeled CO₂ was trapped and quantified. The means \pm SEM is given as absolute quantities of 12 parallels (3 individual experiments with 4 parallels each).

Figure 3.26 shows a trend towards increasing effects of tAdn on oleic acid oxidation. But no statistically significant changes were found.

Acid Soluble Metabolites (ASM) concentration

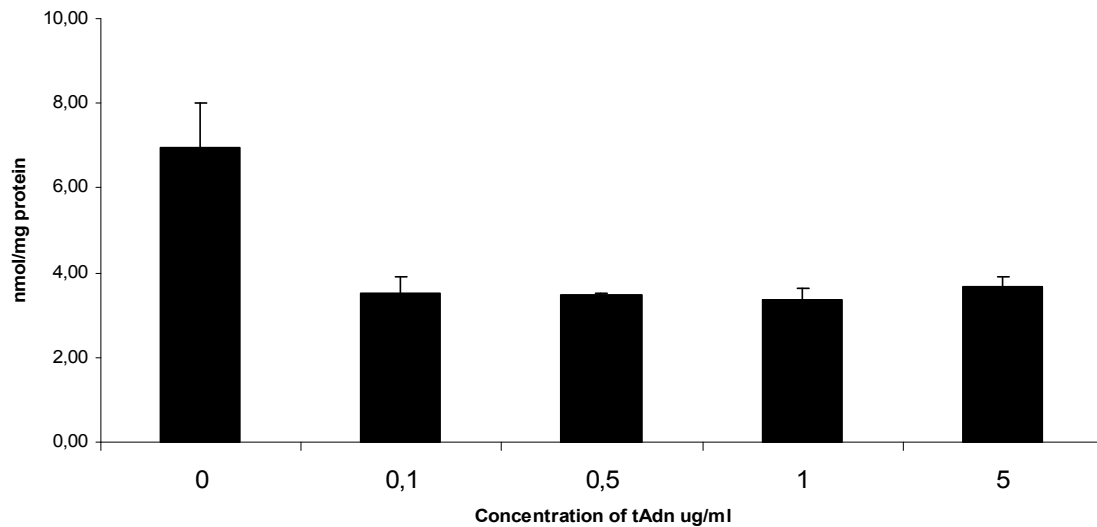


Figure 3.27 Effect of acute incubation with trimer adiponectin (tAdn) on oleic acid oxidation and acid soluble metabolites. Differentiated myotubes were incubated for 4 h with [$1\text{-}^{14}\text{C}$]oleic acid (1 $\mu\text{Ci/mL}$, 50 μM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). Medium tAdn concentrations were 0 (control), 0.1, 0.5, 1 and 5 $\mu\text{g/mL}$. The means \pm SEM is given as absolute quantities of 12 parallels (3 individual experiments with 4 parallels each).

Figure 3.27 shows a trend for tAdn to decrease oleic acid oxidation by approximately 50 % compared to cells not receiving adiponectin. The reduction in oleic acid oxidation were close to significant, p values being 0.06 for myotubes incubated with 0.1 and 1 $\mu\text{g/mL}$ tAdn, 0.09 with 0.5 $\mu\text{g/mL}$ tAdn and 0.07 with 5 $\mu\text{g/mL}$ tAdn.

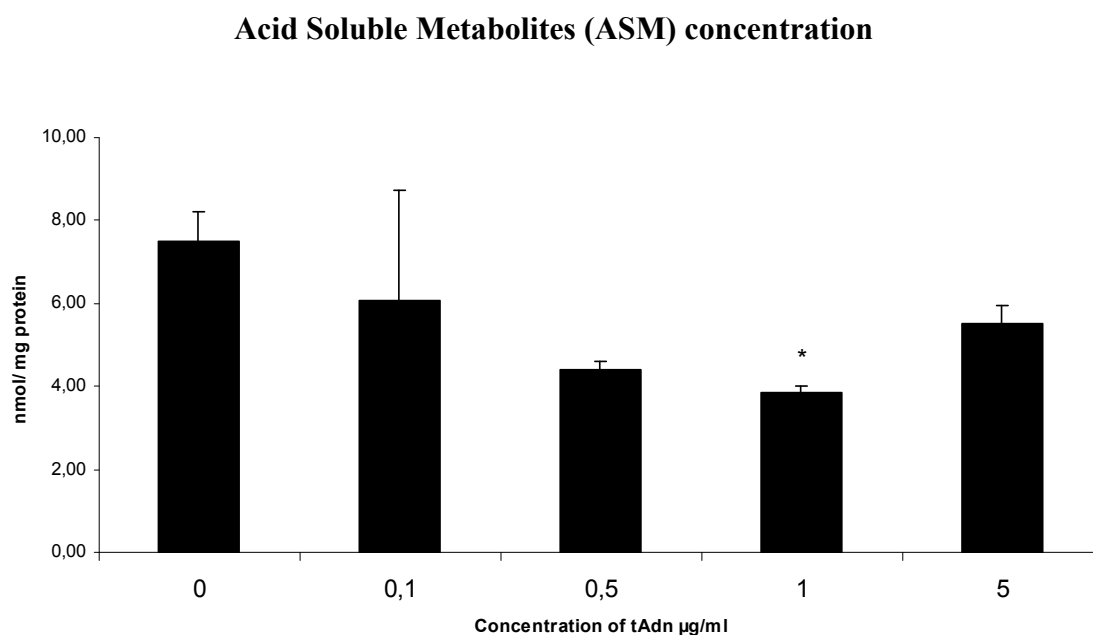


Figure 3.28 Effect of 24 h pre-incubation with trimer adiponectin (tAdn) on oleic acid oxidation and acid soluble metabolites. Differentiated myotubes were pre-incubated 24 h with medium containing 0 (control), 0.1, 0.5, 1 and 5 µg/mL tAdn. Myotubes were then incubated for 4 h with [$1\text{-}^{14}\text{C}$]oleic acid (1 µCi/mL, 50 µM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). Means \pm SEM are given as absolute quantities of 12 parallels (3 individual experiments with 4 parallels each). Asterisk (*) indicates significant oleic acid oxidation change compared to control ($P \leq 0.05$, paired student t-test).

A medium concentration of 1 µg/mL tAdn significantly decreased oleic acid oxidation by 48 % ($P \leq 0.05$) compared to myotubes pre-incubated without tAdn (figure 3.28). Other tAdn concentrations (0.1, 0.5 and 5 µg/mL) seemed to decrease oleic acid oxidation, but these reductions were not statistically significant.

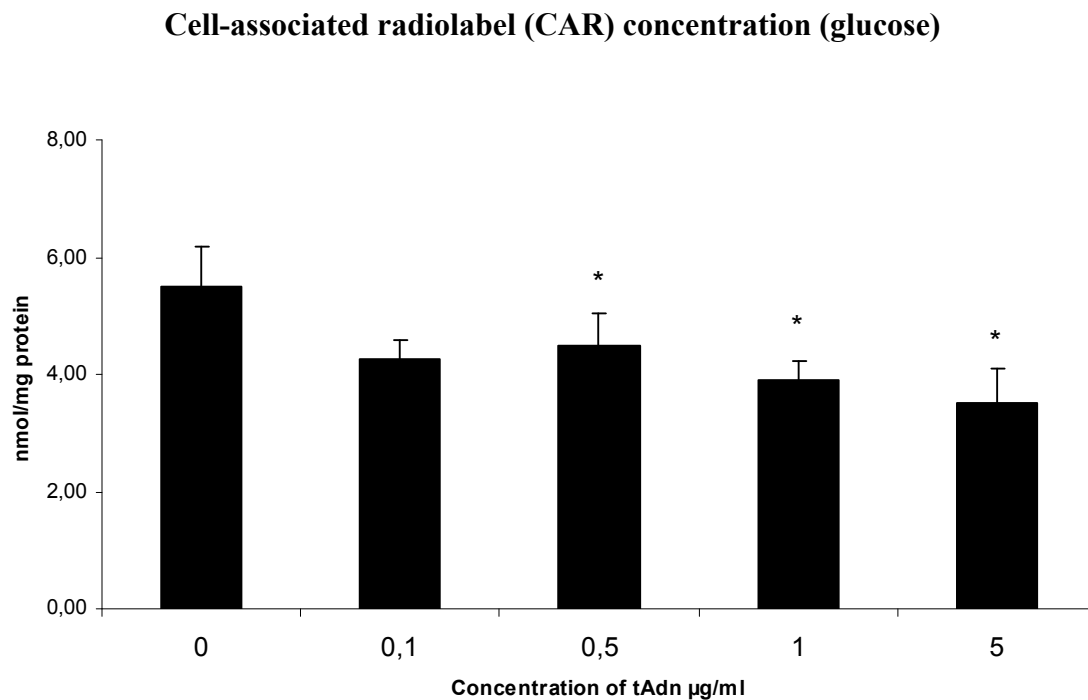


Figure 3.29 Effect of acute incubation with trimer adiponectin (tAdn) on Cell-associated radiolabel and glucose oxidation. Differentiated myotubes were incubated for 4 h with $[6\text{-}^{14}\text{C}]$ glucose (1 $\mu\text{Ci/mL}$, 100 μM) and 0.2 % Fetal Calf Serum (FCS). Medium tAdn concentrations were 0 (control), 0.1, 0.5, 1 and 5 $\mu\text{g/mL}$. Means \pm SEM are given as absolute quantities of 12 parallels (3 individual experiments with 4 parallels each). Asterisk (*) indicates significant CAR change compared to control ($P \leq 0.05$, paired student t-test).

Trimer adiponectin concentrations of 0.5, 1 and 5 $\mu\text{g/mL}$, significantly reduced CAR values by 19, 29 and 36 % ($p \leq 0.05$) respectively (figure 3.29).

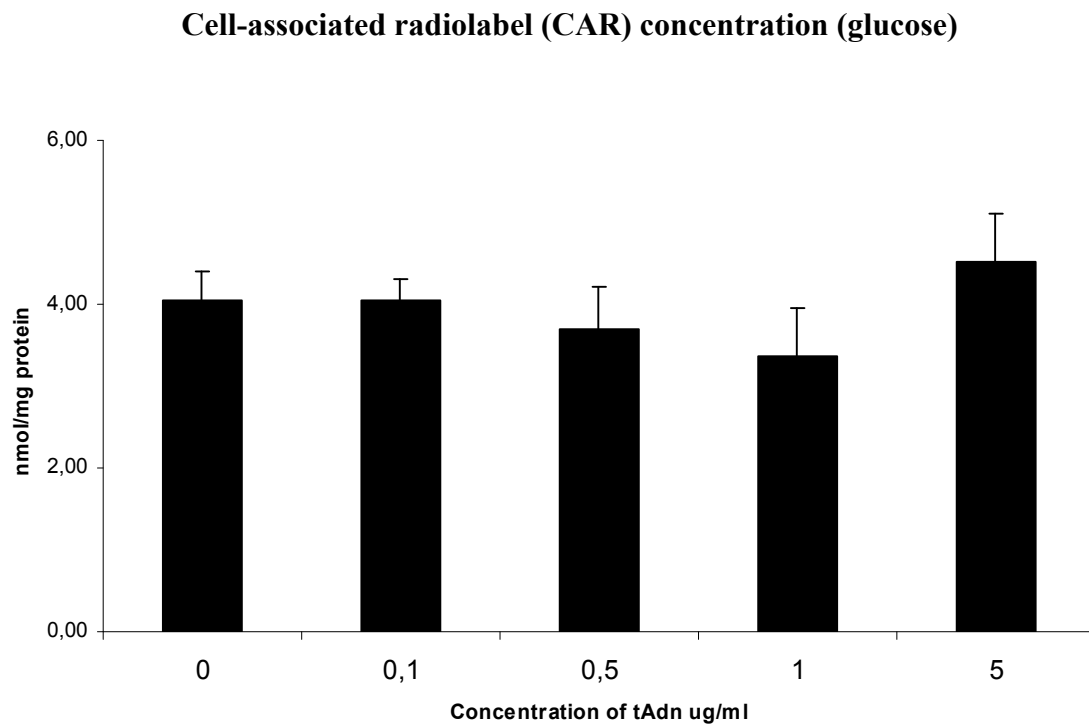


Figure 3.30 Effect of 24 h pre-incubation with trimer adiponectin (tAdn) on cell-associated radiolabel and glucose oxidation. Differentiated myotubes were pre-incubated 24 h with medium containing 0 (control), 0.1, 0.5, 1 and 5 $\mu\text{g/mL}$ tAdn. Myotubes were then incubated for 4 h with $[6\text{-}^{14}\text{C}]$ glucose (1 $\mu\text{Ci/mL}$, 100 μM) and 0.2 % Fetal Calf Serum (FCS). During the 4 hour incubation the medium did not contain tAdn. Means \pm SEM error bars are given as absolute quantities of 12 parallels (3 individual experiments with 4 parallels each).

No effects of tAdn were observed on CAR when pre-incubating myotubes for 24 h before 4 h incubation with labeled glucose (figure 3.30).

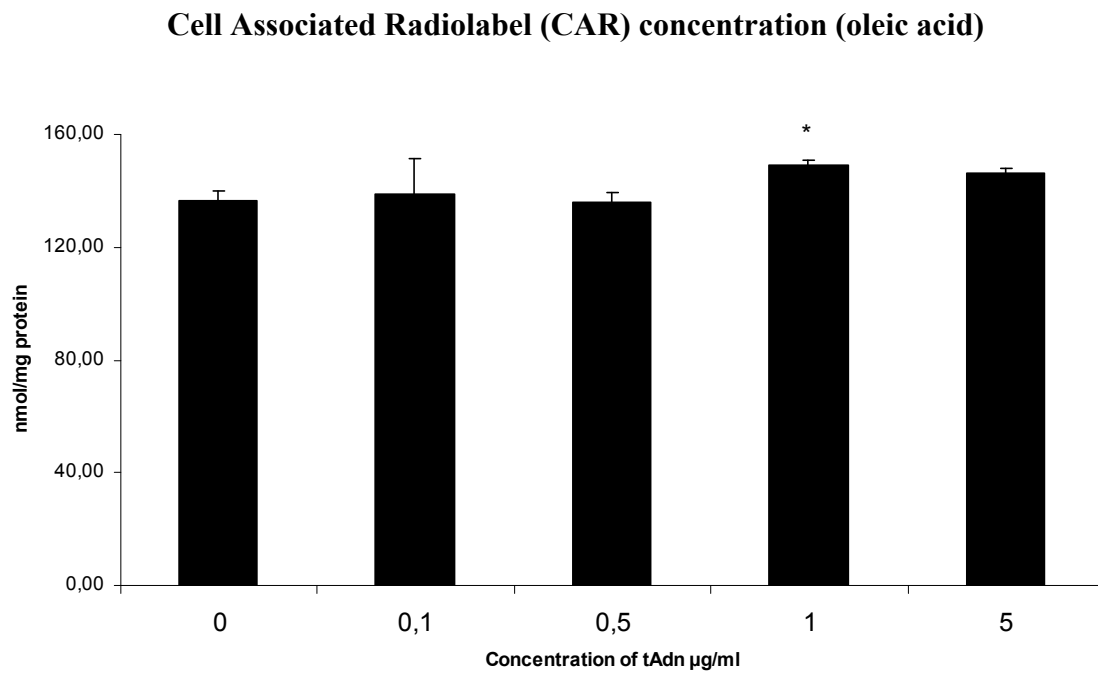


Figure 3.31 Effect of acute incubation with trimer adiponectin (tAdn) on cell-associated radiolabel and oleic acid oxidation. Differentiated myotubes were incubated for 4 h with [$1\text{-}^{14}\text{C}$]oleic acid (1 $\mu\text{Ci/mL}$, 50 μM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). Medium tAdn concentrations were 0 (control), 0.1, 0.5, 1 and 5 $\mu\text{g/mL}$. Means \pm SEM are given as absolute quantities of 12 parallels (3 individual experiments with 4 parallels each). Asterisk (*) indicates significant oleic acid and CAR change compared to control ($P \leq 0.05$, paired student t-test).

A concentration of 1 $\mu\text{g/mL}$ tAdn significantly increased measured CAR values by 9 percent ($p \leq 0.035$) compared to the myotubes incubated without adiponectin (figure 3.31). For the other tAdn concentrations no significant CAR changes were found.

Cell Associated Radioactivity (CAR) concentration (oleic acid)

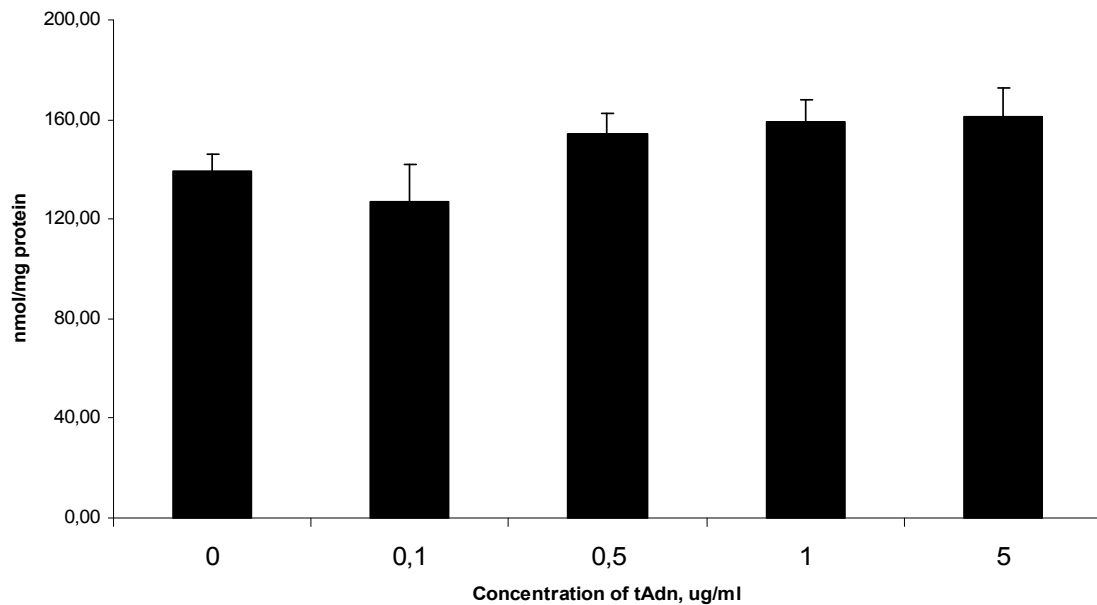


Figure 3.32 Effect of 24 h pre-incubation with trimer adiponectin (tAdn) on cell-associated radiolabel and oleic acid oxidation. Differentiated myotubes were pre-incubated 24 h with medium containing 0 (control), 0.1, 0.5, 1 and 5 $\mu\text{g/mL}$ tAdn. Myotubes were then incubated for 4 h with $[1-^{14}\text{C}]$ oleic acid (1 $\mu\text{Ci/mL}$, 50 μM), 0.2 % Fetal Calf Serum (FCS), L-carnitine (1 mM) and glucose (5 mM). During the 4 hour incubation the medium did not contain tAdn. Means \pm SEM error bars are given as absolute quantities (n=12) (3 individual experiments with 4 parallels each).

As seen in figure 3.32, there seemed to be a tendency for increase in oleic acid oxidation with increasing adiponectin concentration. However, these apparent dose response changes were not statistically significant.

4. Discussion

4.1 Effect of FAs on HepG2 cell AR1/2 mRNA expression

In this study the amount of adiponectin receptors were significantly reduced by saturated FAs in most incubations for AR1, but not AR2. No coherence regarding concentration of FA and reduction of AR was found. A significant reduction in AR for the lowest concentration of FA was not observed for the higher FA concentration. Earlier experiments with HepG2 cells incubated with palmitic acid (16:0) for 24 h had shown a possible reduction in adiponectin receptor mRNA (19;20). This is consistent with the findings of Sun et al that the amounts of AR2 were increased, but not the amounts of AR1. The insulin-sensitizing activities of PPAR γ agonists are associated with their ability to decrease circulating levels of free FAs through inhibition of adipocyte lipolysis, as well as regulation of proteins (adiponectin receptor 1 and 2) that modulate insulin sensitivity and lipid metabolism (22).

The reduction in expression of AR 1 and 2 mRNA was more evident for HepA compared to the other FAs (figure 3.4). All FAs used in these experiments were saturated with increasing carbon chain length. There seemed to be no correlation between increasing FA carbon chain length and decreasing AR mRNA expression. No published experiments with HepG2 cells and the FAs used were found, making comparison with other experiments difficult.

The FA analogue tetradecylthioacetic acid (TTA) was found to inhibit secretion of triacylglycerol from rat hepatocytes, mainly by stimulating FA oxidation and then promote hypotriglyceridemia (23). The 3-thia FAs increase FA oxidation in liver through inhibition of malonyl-CoA synthesis, activation of carnitine palmitoyl transferase I (CPT I), and induction of carnitine palmitoyl transferase II (CPT-II) and enzymes of peroxisomal β -oxidation. Activation of FA oxidation is the key to the hypolipidemic effects of 3-thia FAs. Also other lipid metabolizing enzymes are induced. The mechanism of enzyme induction seen with TTA is not completely understood, but the peroxisome proliferator activated receptor (PPAR α) may be involved (24). From our experiments, no significant reduction in the expression of AR1 or AR2 mRNA were observed when liver cells were incubated for 24 h with the FA analogues at three different concentrations (50, 100 or 200 μ M). This is

consistent with what has been published regarding TTAs metabolic effects on liver cells (23;24).

Studies have shown that FAs may participate in the regulation of gene expression. The exact mechanisms (specific binding to various nuclear receptors or changes in the abundance of regulatory transcription factors) by which FAs exert their effects are not fully understood, but non-esterified FAs or their CoA derivatives seem to be the main signals involved in the transcriptional effect of long-chain FAs (25).

Several of the experiments done with expression of adiponectin receptor regulation have been performed on mice and rats feed high fat diets rich in saturated FAs. The only modification of adiponectin receptor expression observed when feeding rats high fat diets rich in saturated FAs, was a decrease in liver AR2 mRNA concentrations (14). Adiponectin receptor expression in rats is poorly responsive to modification of nutritional conditions and this expression was not decreased in a genetic model of insulin resistance (14). Neumeier et al found that in fasted mice, the AR1 and AR2 mRNA were induced in the liver, and re-feeding decreased the expression. This suggests that a reduced expression of adiponectin receptors may contribute to fatty liver disease in obesity and metabolic syndrome (26). However, these results do not indicate a regulation of adiponectin receptors by FAs, and no published studies have been performed with these FAs regarding effect on the expression of adiponectin receptors in humans. Staiger et al tested whether there is a correlation between AR1 mRNA expression and plasma free fatty concentrations. No association could be found by simple and multivariate linear regression analysis (27).

The expression of adiponectin receptors is regulated by different pathways in different cell types, and regulation is likely to be associated with altered glucose and lipid metabolism in either T2D cells or after incubation with antidiabetic drugs (22). Functional involvement of the glucocorticoid receptor (GR) element in the regulation of adiponectin receptor 2 in liver cells has been shown. The increased expression of AR2 is expected to lead to an increased sensitivity of liver cells to adiponectin and thereby to enhancement of the antidiabetic activity of adiponectin (22). Sun et al found that the PPAR γ agonist rosiglitazone was able to elevate both the mRNA and protein levels, as well as to stimulate promoter activity of adiponectin receptor 2, but not 1 in HepG2 cells (22).

Kadowaki et al found that the levels of AR1 and AR2 mRNA expression in the liver and skeletal muscle increased after fasting, and refeeding rapidly restored these to levels equal to the original fed state (8). The amounts of FAs in plasma were increased after fasting. This study was based on a 24 hour experiment, and if the changes in gene expression were induced rapidly, this would not be revealed. The effect of FAs on longer time intervals was not measured in this experiment. Similar problems were seen in our experiments. This was the same challenges with the experiments performed in our study.

The distribution of different adiponectin receptors varies greatly, as have been described in other studies. In these experiments we have just looked at HepG2 cell mRNA expression, which can be different in other hepatic cell lines, primary cells and cells *in situ*. Further experiments are needed to investigate the tendency of saturated FAs to reduce the amounts of AR1 and 2 mRNA to draw any clear conclusions.

4.2 T-cadherin, adiponectin receptor 3

T-cadherin was most abundantly expressed in fetal brain, heart, uterus, lung and skeletal muscle (fig 3.8). The result of our analyzes was consistent with other findings, where Bromhead et al found that T-cad was expressed widely in the brain, the cardiovascular system and also in lung and heart (10). The distribution of T-cad was distinct from the distribution of AR1 and AR2. AR1 mRNA was relatively highly expressed in skeletal muscle, but even higher in other tissues like testis, placenta and fetal liver. AR2 mRNA was highly expressed in liver, both fetal and adult (19;20). AR1 and AR2 are GPI anchored receptors compared to T-cad, which is an extracellular receptor. AR3 was first identified as a cadherin receptor, and later as an adiponectin receptor.

The amount of T-cad mRNA was measured quantitatively with both real-time PCR and by semi-quantitative methods like western blotting in the study by Bromhead et al (10). The amount of T-cad mRNA was measured quantitatively only by real-time PCR in our experiment.

The tissue distribution of T-cad was found after analyzing only one single tissue panel from ACCT. To get a more certain picture of the T-cad distribution in the body we need to analyze other tissue panels and see if they display the same results since there may be inter

individual variations in distribution of T-cad. Adiponectin receptors have selective affinity to the amount and different types of adiponectin in plasma. T-cad is a receptor for hexameric and high-molecular-weight (HMW) forms of adiponectin, but not for the trimeric and globular adiponectin (10). Based on the assumption that there is an inverse correlation between plasma levels of adiponectin and T-cad, this may explain the low amounts of T-cadherin receptors since it binds HMW adiponectin and almost all adiponectin appears to exist as full-length adiponectin in plasma (8). If this assumption is correct, low amounts of receptors are needed to exert effects of adiponectin.

The objective for these experiments was to analyze the effect of FAs on T-cad mRNA expression in HepG2 cells, as had been done for AR1 and AR2. Due to low amounts of T-cad receptor in these cells, this was not possible. The low amounts of T-cad in liver were confirmed when we looked at the results from the tissue panel analysis, (fig 3.8). Lee (1996) found that T-cad is hardly detectable in the liver (28) which is consistent with our finding. Further experiments with HepG2 cDNA and the expression of T-cadherin receptor were not performed.

4.3 Human myotubes

Globular adiponectin seems to increase the amount of oleic acid oxidation more than full-length adiponectin and trimer adiponectin in human myotubes in our experiments. Civitarese et al. found that adiponectin incubation increases mitochondrial bioenergetics in primary human myotubes (12). Two days incubation with 0.25 and 0.5 $\mu\text{g/mL}$ of gAdn increased mtDNA content (12). In our experiments the preincubation with gAdn did not significantly increase FA oxidation in human myotubes compared to the control cells incubated with no gAdn (fig 3.16 to 3.21). Yamauchi et al. have shown that 2-week administration of full-length or globular head domain of adiponectin to mouse models of obesity and lipoatrophy, reversed insulin resistance associated with both lipoatrophy and obesity. Also, chronic infusion of a very low dose of gAdn in mice consuming a high-fat and high-sucrose diet prevented development of obesity without affecting food intake (29). But these results can not be generalized directly to human myotubes.

Yoon et al. showed that adiponectin increases FA oxidation via activation of AMPK and phosphorylation of ACC. However, the phosphorylation of ACC induced by adiponectin is

short-lived (30). Therefore, this pathway may not fully explain the long-term effects of adiponectin on weight loss and FA oxidation. On the other hand, activation of PPAR α would be a promising pathway by which adiponectin can trigger long-term stimulation of FA oxidation, even when initial signaling is not present. This would probably increase the amounts of relatively long-lived enzymes involved in FA metabolism (30). PPAR α is a ligand-activated nuclear hormone receptor that is highly expressed in tissues that obtain most of their energy from FA oxidation, including liver, heart, kidney and skeletal muscle. PPAR α controls the expression of a number of genes involved in peroxisomal and mitochondrial β -oxidation, such as ACO (acyl-CoA oxidase), CPT1 and FABP3 (fatty acid binding protein 3) (30). Expression of PPAR α in the myotubes used in our experiments was not analyzed.

There are few published experiments testing the effect of different types of adiponectin on myotubes and fatty acid (FA) oxidation. We expected to observe an increase in β -oxidation in the myotubes, at least for the experiments with globular adiponectin since adiponectin receptor-1 is abundantly expressed in skeletal muscle and is a high-affinity receptor for globular adiponectin (31). Adiponectin receptor-2 expression is restricted to muscle and liver and has intermediate affinity for globular and full-length adiponectin. Previous studies have shown differences in biological activity between globular and full-length adiponectin (32). A proteolytic cleavage product of adiponectin that includes its globular head group (gAdn), induces FFA oxidation and glucose uptake in muscle, whereas full-length adiponectin has been shown to inhibit hepatic glucose production (32).

It is well established that AMP-activated protein kinase (AMPK) in skeletal muscle cells increases FA oxidation by inhibiting acetyl-CoA carboxylase (ACC) through phosphorylation. A decrease in ACC activity reduces intracellular malonyl-CoA. Since malonyl-CoA is an allosteric inhibitor of carnitine palmitoyl transferase 1 (CPT1), a repressed production will increase CPT1 activity. This ultimately increases influx of long-chain FAs into the mitochondria where they are oxidized (30). This could not be observed on our experiments.

The CO₂ trapping method used in our experiments with myotubes is described in a recent paper by Wensaas et al. (33). The measurement of acid soluble metabolites (ASM), cell-associated radioactivity (CAR) and CO₂ are well established methods. It is generally

accepted to use ASM as a measure of FA oxidation, alone or in parallel with CO₂ measurements (34). Direct measurements of CO₂ production were done in parallel with ASM production, as a measure of FA oxidation. CO₂ measures the flux into Krebs cycle, whereas the sum of CO₂ and ASM measures total fatty acid oxidation.

No effect on glucose oxidation or uptake was seen for the myotubes preincubated with tAdn and later incubated with labeled glucose medium. A significant increase was observed in CO₂ measurements for myotubes incubated with 1 µg/mL tAdn and glucose labeled medium (fig 3.23) and for CAR measurements from the same experiment at 0.5, 1 and 5 µg/mL (fig 3.29). The reason for the different results could be that the effect of tAdn on glucose metabolism could be short lasting. Since only two pre-incubation times were investigated, there was no information regarding when the onset of the prospective maximum rate of glucose metabolism happens and when a prospective decrease begins. More experiments are needed to reveal effects of tAdn concentrations between 1 and 5 µg/mL to assess dose-response effects properly. No studies were found having performed comparable experiments with tAdn.

Bruce et al found that administration of the globular head of adiponectin (1 h incubation together with [1-¹⁴C]palmitate) reduces plasma glucose levels and ameliorates insulin resistance in mice (31). Recent studies have also shown that incubation of isolated rodent skeletal muscle with globular adiponectin stimulates glucose uptake (31). Globular adiponectin increases glucose uptake in the absence of insulin in skeletal muscle from both lean and obese individuals (31). It has been reported that adiponectin increases glucose uptake in skeletal muscle, apparently via activation of AMPK. It remains to be determined whether adiponectin increases GLUT4 translocation in skeletal muscle or not (35). Ceddia et al investigated the effect of globular adiponectin (gAdn) on GLUT4 translocation, glucose uptake, glycogen synthesis, glucose oxidation, lactate production and FA oxidation in muscle cells in culture (35). They found that gAdn increases glucose uptake in skeletal muscle cells via GLUT4 translocation and subsequently reduces the rate of glycogen synthesis and shifts glucose metabolism toward lactate production. These effects are consistent with the increased phosphorylation of AMPK and ACC and oxidation of FAs induced by globular adiponectin (35). More experiments are needed to assess long-term effects on dose-response of tAdn on glucose oxidation.

Only a few experiments with trimer adiponectin have been published, making it difficult to compare our experiment with similar findings. One experiment used skeletal muscle in rats. Tsao et al. showed that only trimeric adiponectin, not hexameric or HMW adiponectin isoforms activated AMPK in skeletal muscle from rats (36).

Species difference between human myotubes and rodents and inter-individual variation in tissues examined, may explain the difference between our results and published literature where adiponectin significantly increased FA oxidation. The method used in these experiments is relatively new. The use of different methods may contribute to non-existing variation. Myotubes or primary muscle cells from three different donors were used: lean, obese and obese type 2 diabetic. The amounts of adiponectin receptors in the myotubes from these donors were not measured, although this would be an interesting variable to investigate further. The results from our experiments were calculated by using paired t-test. Too few experiments were performed to assess any effect of tAdn.

5. Conclusion and future perspectives

Experiments with HepG2 cells

This study showed that incubating HepG2 cells with saturated fatty acids (FAs) had a tendency to reduce the amounts of adiponectin receptor 1 (AR1). The results showed inconsistency with respect to increasing concentrations of FA and a further reduction in AR expression levels. For AR2 a significant reduction was seen only when preincubating HepG2 cells with 50 μ M stearic acid. The FA analogues (TTA, α TTA TTTA) did not alter the expression of AR in HepG2 cells. Due to inconsistent results with respect to concentration of FA and the reduction of AR1 expression levels, further experiments are needed to assess whether FA reduces the expression of adiponectin receptors.

FA can influence the effect of adiponectin in other ways than just downregulation of the adiponectin receptors. The regulation could be downstream of the receptor. The amount of AMPK or other intracellular signal substances should be investigated to see if FA incubation results in any change. Expression of the human AR1 and AR2 genes may be different in other hepatic cell lines, primary cells and cells *in situ*. It has also been emphasized that transcription studies in cell cultures do not necessarily reflect processes *in vivo*.

Expression of T-cadherin

T-cad was mostly expressed in fetal brain, heart, uterus, lunge and skeletal muscle and hardly detectable in liver. The amounts of T-cad in malign lymphoid cell lines were 100 to 1000 fold lower than the amounts in myotubes. The distribution of T-cad was distinct from the distribution of AR1 and AR2. More studies are needed to survey the effect downstream of T-cad.

T-cad as an adiponectin receptor (AR3) was discovered in 2004, and is thus relatively new. This receptor is different from AR1 and AR2 and the tissue distribution also differs. Since the amounts of receptor are relatively low in liver it may be challenging to study down-regulation by different substances similar to the experiments done with AR1 and AR2.

Human myotubes and adiponectin

Incubation of human myotubes with adiponectin showed inconsistency with respect to the effect on FA oxidation. Full-length adiponectin, globular adiponectin and trimeric adiponectin were used. Globular adiponectin seemed to increase the amount of oleic acid oxidation more than full-length adiponectin and trimer adiponectin. Trimer adiponectin seemed to increase the amount of glucose oxidation. More experiments are needed to assess the effect of adiponectin on fatty acid and glucose oxidation.

Adiponectin is a potent bioactive substance, with the ability to increase FA oxidation in human myotubes. This is seen for globular adiponectin in several studies. The effect of trimer adiponectin has not been investigated as thoroughly. The amounts of adiponectin receptors should be investigated in the myotubes. Human myotubes is suited for these experiments since the amount of adiponectin receptors are found to be relatively high. The CO₂ trapping method is suited for these types of metabolic experiments, and thus should be considered as an appropriate system for future experiments.

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Appendix

Culture medium for HepG2 cells

500 ml MEM medium (Sigma, #M2279)

50 ml heat inactivated fetal calf serum (FCS) (Sigma, #F7524)

5 ml penicillin –streptomycin (sigma, #P4333, 10000 U/mL penicillin, 10 mg/mL streptomycin)

5 ml L-glutamine (Sigma, #G7513)

5 ml non-essential amino acid solution (Sigma, #M7145, 100x)

5 ml sodium-pyruvate (Sigma, #S8636, 11 mg/mL)

Making FAs stock, for experiments with HepG2 cells

Bovine serum albumin solution (BSA), without fatty acids.

Mw 67.000, 2.5 mM concentration

Taking the average value for the different FAs, approximately 67 μ l

161 mg BSA solved in 933 μ l DMEM, 8 g BSA in 46.65 ml DMEM.

Making of fatty acid solutions

Final concentration 6mM in bovine serum albumin (BSA)

0.025 g of each FA was solved them in DEPC water. As a sodium salt the FAs were easily solved, otherwise they were solved in 0.1M NaOH.

The suspensions were heated in a water bath holding 96°C. BSA was heated to 50°C and 5 ml BSA added to each of the FAs and mixed thoroughly. The final concentration of the different FAs was 6 mM. When the liquid were clear they were sterile filtered and batched out in 2 ml plastic tubes. Store at -20°C.

Before the FAs were added to the HepG2 cells they were diluted with DMEM medium to concentrations of 200, 100 and 50 μ M.

The wells were added FAs and let to stand 24 h before they were harvested.

Bovine Serum Albumin 6 %, BSA

Measure up 1.2 g bovine serum albumin with FAs in to a 50 ml plastic tube. Add 18 ml milliQ water. Turn the tube to dissolve the powder. If much foam, spin down

Medium for human skeletal muscle cells

M0- medium:

500 ml DMEM low glucose (Sigma, #D6046)

10 ml FCS (Fetal Calf Serum, not heat inactivated, Sigma #F7524) -2 %

5 ml Penicillin/Streptomycin (Gibco BRL, #15140-114, 100ml)

M1- seeding:

500 ml DMEM low glucose (Sigma, #D6046)

50 ml FCS (Fetal Calf Serum, not heat inactivated, Sigma #F7524) -10 %

5 ml Penicillin/Streptomycin (Gibco BRL, #15140-114, 100ml)

(Take out 25 ml from M0-medium and add 2 ml FCS)

M2- Proliferation:

500 ml DMEM low glucose (Sigma, #D6046)

10 ml FCS (Fetal Calf Serum, not heat inactivated, Sigma #F7524) -2 %

10 ml Ultrosor-G, UG (Bio Serpa, #15950-017)

5 ml Penicillin/Streptomycin (Gibco BRL, #15140-114, 100ml)

(Take out 200 ml from M0-medium and add 4 ml UG)

M3- Differentiation:

500 ml DMEM low glucose (Sigma, #D6046)

10 ml FCS (Fetal Calf Serum, not heat inactivated, Sigma #F7524) -2 %

5 ml Penicillin/Streptomycin (Gibco BRL, #15140-114, 100ml)

25pmol Insulin (21 µl of 1000 x diluted Insulin Actrapid, Novo Nordisk 0.06 mM)

(Take out 24 ml from M0-medium and add 1 µl 25pmol/µl Insulin)

Ultrosor-G (Bio Serpa, #15950-017) should be re-constituted by adding 20 ml MQw to the container. Wait for 15 min, sterile filtrate and transfer 10 ml to the M2- medium. The rest is frozen (-20°C) for further use.

Making radioactive oleic acid, 0.1 μ Curie/ μ l

Take out 50 μ l radioactive oleic acid in ethanol. Use N₂ gas to evaporate the ethanol to a volume of 5-10 μ l. Add 20 μ l 0.01 M NaOH, vortex 1-2 min. Add 16 μ l BSA (2.4 mM in PBS) and vortex 1 minute. Heat for 15-30 min, vortex and spin down. Transfer to plastic tube which contain 5 ml sterile filtrated PBS. Add 5 μ l l-carnitine (1M) and 25 μ l glucose (1 M).

Making Luria-Bertani (LB) medium and LB agar

Mix 10 g peptone, 5 g yeast extract and 10 g NaCl in a 1 liter glass bottle. Add approximately 950 ml MilliQ water. Adjust the pH to 7.0 at 23°C, with NaOH 1 M. Adjust the volume to 1 liter. Autoclave the liquid. Cool down to 55°C and add antibiotics. Store at room temperature or 4°C.

Make 1 liter LB medium, adjust the pH to 7.0 and add 15 g agar. Autoclave. Cool down to 55 °C and add Kanamycin to the concentration (50 μ g/ μ l). Pour 20 ml of the liquid over 10 cm Petri dishes. Let the liquid stiffen, turn the dishes upside down and store at 4°C.

Calculations

AR1: The vector is 1127 bp + 2580 bp = 3707 bp

The molecule weight per bp = 660 g/mol

3707 bp x 660 g/mol = 2446620 Mw

Standard for AR1 $10^{-13}/2\mu\text{l} \times 6.022 \times 10^{23} = 24614$ copies/2 μ l

Mw AR1

AR2: The vector is 1160 bp + 2580 bp = 3740 bp

The molecule weight per bp = 660 g/mol

3740 bp X 660 g/mol = 2468400 Mw

Standard for AR2 $10^{-13}/2\mu\text{l} \times 6.022 \times 10^{23} = 24396 \text{ copies}/2 \mu\text{l}$

Mw AR2

T-Cad/AR3: The vector is 2141 bp + 2580 bp = 4721 bp

The molecule weight per bp = 660 g/mol

4721 bp X 660 g/mol = 3115860 Mw

Standard for AR3 $10^{-14}/2\mu\text{l} \times 6.022 \times 10^{23} = 1933 \text{ copies}/2 \mu\text{l}$

Mw AR3

RPL27: The vector is 410 bp + 2580 bp = 2990 bp

The molecule weight per bp = 660 g/mol

2990 bp X 660 g/mol = 1973400 Mw

Standard for RPL27 $10^{-11}/2\mu\text{l} \times 6.022 \times 10^{23} = 3051586 \text{ copies}/2 \mu\text{l}$

Mw RPL27